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THE ROLE OF THE OVIDUCT IN EMBRYONIC SURVIVAL IN THE GILT

by

SUSAN NOVAK ©

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1517 Chemin de la Grande Ligne
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G2N 1Z8

Dated : Sept 6, 2000

ABSTRACT

The potential role of the oviduct in nutritionally-mediated, and locally regulated effects on embryonic survival was investigated. In the first experiment, time after ovulation was correlated (r=0.79, P=0.0001) with peripheral, but not oviductal, progesterone concentrations suggesting that nutritional effects on peripheral progesterone are temporally different to effects within the ovarian and oviductal vasculature. Two experiments established temporal and steroid-dependent changes in the oviduct during the peri-ovulatory period. Temporally, protein and insulin-like growth factor (IGF)-I concentrations in oviduct flushings decreased (P<0.01), and porcine oviductal secretory protein (POSP) mRNA and protein abundance was lowest (P = 0.001) on d 2 after ovulation, coincident with changes (P=0.0001) in plasma progesterone concentrations and the estrogen: progesterone (E:P) ratio. Unilaterally ovariectomized gilts were used to study local steroid dependent regulation of oviduct function. Plasma steroids were higher (P<0.001) in oviductal veins ipsilateral (INT) than contralateral (OVX) to the remaining ovary, confirming sub-ovarian countercurrent transfer of steroids in vivo. Protein concentration was higher (P=0.031) and POSP abundance was lower (P=0.0001) in OVX than INT oviductal flushings. Two final experiments determined whether nutritionally-induced changes in embryonic survival could be mediated by oviduct function. Using an established experimental paradigm that results in differences in embryonic survival and progesterone status in the immediate post-ovulatory period, gilts were restrict fed in the first (RH) or second week (HR) of the estrous cycle. In this study, protein concentration increased ($P=0.002$) in oviduct flushings, coincident with a higher ($P<0.05$) E:P ratio in peripheral plasma in the RH group. The second study used the same nutritional paradigm, plus a third insulin-treated group. HR had lower ($P<0.05$) E:P ratio, estradiol, and IGF-I concentrations in oviductal plasma, lower protein and IGF-I concentrations, and POSP abundance in oviduct flushings ($P<0.01$), and lower ($P<0.05$) POSP and IGF binding protein (IGFBP)-4 mRNA in oviduct tissue compared to RH. Insulin treatment differentially affected the oviduct environment by lowering ($P=0.0001$) POSP abundance, and increasing ($P=0.026$) IGFBP-4 mRNA compared to HR gilts. Previous feed restriction and insulin treatment affects the oviduct environment and may contribute to nutritional effects on embryonic survival.

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **THE ROLE OF THE OVIDUCT IN EMBRYONIC SURVIVAL IN THE GILT** submitted by **SUSAN NOVAK** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **ANIMAL SCIENCE**.

George Foxcroft

Walter Dixon

Sandra Davidge

Ronald Ball

Fred Menino

Dated: Sept 6, 2000

Dedicated to my husband,

Marty Budnyk

for his support, understanding, and encouragement

All of my love,

Susan

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LIST OF ABBREVIATIONS

^{3}H	Tritium
^{51}Cr	Radiolabelled chromium
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BW	Body weight
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine monophosphate
CCAC	Canadian Council on Animal Care
cDNA	complimentary deoxyribonucleic acid
Cl^{-}	Chloride ion
CP	Crude protein
CV	Coefficient of variance
D1	Dopamine type 1
DE	Digestible energy
E:P ratio	Estrogen:Progesterone ratio
EGF	Epidermal growth factor
EGP or EAP	Estrus-associated glycoprotein
EtOH	Ethanol
FGF	Fibroblast growth factor
GH	Growth hormone
hCG	human chorionic gonadotropin
HCO_3^-	Bicarbonate ion
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
INT	Oviduct ipsilateral to remaining ovary

LIST OF ABBREVIATIONS (CONT'D)

K^+	Potassium ion
LH	Lutenizing hormone
LIF	Leukemia inhibitory factor
LSM	Least squares mean
M; Maintenance	Feeding to energy maintenance, calculated at 461 MJ DE/kg metabolic BW (BW $kg^{.75}$)
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
Na^+	Sodium ion
NRC	National Research Council
OGP	Oviduct glycoprotein
OVX	Oviduct contralateral to the remaining ovary
PA	Plasminogen activator
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
POSP	Porcine oviductal secretory protein
RIA	Radioimmunoassay
RT-PCR	Reverse transcriptase-polyclonal chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBS-T	Tris buffered saline-0.1% Tween 20
TGF	Transforming growth factor
TIMP	Tissue inhibitor of matrix metalloproteinases
ZP	Zona Pellucida

GENERAL INTRODUCTION

Commercial pig production is very intensive and increased emphasis has been placed on the reproductive capabilities of gilts and sows. This includes minimizing non-productive days, and increasing the number of pigs weaned per sow per year through attainment of maximum litter size. Another challenge to producers is for the replacement gilts to achieve large litter sizes, as gilts comprise up to 40 % of the reproductive herd. As one approach to achieving such goals, feeding strategies are being developed to maximize ovulation rate and increase embryonic survival.

The maximum attainable litter size is determined by the ovulation rate of the pig. A reduction in potential litter size may then occur around fertilization, in the peri-implantation period, and during fetal growth and development. The majority of the loss occurring post-implantation is due to uterine capacity, as each embryo and associated trophoblast requires a certain amount of uterine space after day 30 of gestation in order to become a live born fetus (Webel and Dziuk, 1974). In sows, most of the prenatal loss may occur after implantation, as ovulation rate and pre-implantation survival can be high relative to gilts, resulting in uterine crowding becoming a major factor (Foxcroft, 1997). In contrast, since gilts have a lower ovulation rate than sows, the majority of embryonic loss occurs in the peri-implantation period, with relatively little loss in the post-implantation phase. Although patterns of embryonic loss are changing in modern genotypes, there is still substantial loss during the pre-implantation period (Pope and First, 1985). The factors that determine embryonic survival between ovulation and implantation are, therefore, of importance in efforts to achieve a high litter size in gilts.

During early pregnancy, the diverse population of embryos develops in a uterine environment largely under the influence of progesterone (see Roberts and Bazer, 1988). Alterations in uterine protein secretion can adversely affect the development of these embryos, which can consequently lead to losses at implantation. Furthermore, the ability of an embryo to successfully implant is a product of the communication between the embryo and the uterus (Roberts et al., 1993), and if communication is asynchronous, it can be a cause of embryonic loss (see review by Pope, 1988). Another important consideration is that more advanced embryos within a litter synthesize estrogens sooner, and preferentially advance the uterine environment to their own benefit; however, this may be detrimental to lesser-developed embryos (Geisert et al., 1982; Xie et al., 1990). Variation in the rate of pre-implantation embryo development within a litter is, therefore, likely another cause of embryonic loss (see review by Pope et al., 1990) and possibly can be traced to follicular heterogeneity (Hunter and Wiesak, 1990).

The oviduct is another potential factor contributing to embryonic loss. Gamete transport and maturation, fertilization, and early embryonic development all occur in the oviduct, and thus the oviduct environment should be appropriate for facilitation of these diverse events. Consequently, changes in the oviductal environment surrounding the gametes and embryos are very dynamic and the regulation of this changing environment is largely dependent on steroids circulating in the maternal blood (Leese, 1988). Early gamete transport and fertilization occur under an estrogen-dominated environment, whereas embryonic transport through the oviduct is largely under progesterone control. Therefore, improper steroid priming of the oviduct may adversely affect fertilization and early embryonic development, and thus contribute to embryonic loss. As a consequence, both the absolute concentrations of ovarian steroids, and the timing of changes in the temporal relationships between estrogen and progesterone are important to embryonic survival (Ashworth, 1991; Pharazyn et al., 1991; Pharazyn, 1992; Blair et al., 1994; Soede et al., 1994; Jindal et al., 1996; Jindal et al., 1997; Almeida et al., 2000a; van den Brand et al., 2000).

Although the oviduct was originally thought of as a passive conduit, evidence is accumulating to suggest a very active role for the oviduct in gamete transport and maturation, fertilization, and early embryonic development (see reviews by

Gandolfi, 1995; Nancarrow and Hill, 1995; Buhi et al., 1997). Evidence for an active role of the oviduct environment is evident from in vitro fertilization and embryo culture studies. Embryonic development in vitro never occurs at the same rate as in vivo conditions, and there is typically a developmental arrest occurring at a species specific stage corresponding to the initiation of expression of the embryonic genome (see Bavister, 1988). However, improvements in embryo development were achieved by culturing embryos with oviductal cells (Gandolfi and Moor, 1987; Xu et al., 1992; Xia et al., 1996; Yeung et al., 1996). The oviductal secretions may aid the activation of the zygote genome (Bavister, 1988; Telford et al., 1994), as passage into the uterus is related to this critical stage in many species. Also, the oviduct fluid is either thought to contain factors that the embryo requires or to remove metabolites that are toxic to the embryo. The beneficial effect of oviduct co-culture on embryonic development is also evident when cross-species co-culture is performed (Yadav et al., 1998), suggesting a commonality between species in oviduct secretions. However, species-specific effects on sperm and egg binding are evident with respect to the roles of certain oviductal glycoproteins (Schmidt et al., 1997). Although in vitro experiments have provided general evidence of a beneficial effect of oviduct secretions on the embryo, the presence of the sub-ovarian countercurrent system (see review by Krzymowski et al., 1990) allows for local ovarian steroids to regulate oviduct function in a very unique way in vivo. The impact of this local level of regulation needs careful consideration when studying how the timing of steroid dependent changes in the oviduct environment may contribute to embryonic survival.

Nutritional manipulation during the estrous cycle of gilts (Ashworth, 1991; Almeida et al., 2000a), and during lactation in sows (Zak et al., 1997), has resulted in effects on subsequent embryonic survival, which can either be attributed to changes in folliculogenesis and oocyte quality, or to alterations in the oviductal or uterine environments (see review by Foxcroft, 1997). Regulation of the uterine environment is currently being investigated in many laboratories, as the majority of embryonic loss occurs during the embryos' residence in the uterus. Many studies have implicated alterations in the oviductal environment as a contributor to embryonic loss (Ahmad et al, 1995; Jindal et al., 1996; Mburu et al., 1998). However, the oviduct environment at this critical time has not been extensively investigated.

The purpose of the research presented in this thesis was firstly to demonstrate that the oviduct environment is responsive to alterations in ovarian steroids in vivo, and then to elucidate a possible role for the oviduct in embryonic survival, by exploiting nutritional models that create differences in embryonic survival. Based on a review of the literature, the first chapter provides the basis for understanding how the oviduct could be involved in embryonic survival. Due to the vast amount of available literature, this review of literature will be focused on livestock species, and specifically the pig, and references are made to other species when appropriate. The first section discusses factors that can contribute to embryonic loss and nutritional models that have been developed to study the mechanisms involved in embryonic loss. The second section provides an extensive background into the properties and functions of the mammalian oviduct, with specific attention to the events occurring in the pig oviduct. The third section presents literature on the role of receptor-mediated action of steroids in regulating oviductal cells and inducing synthesis of oviductal proteins. In the fourth section, the role of the sub-ovarian countercurrent system is reviewed in the context of steroid-mediated changes in the oviductal environment. The functions of the oviduct and components of oviduct fluid in gamete transport and maturation, fertilization and early development are discussed in the last section.

Chapters 2 to 6 detail the experimental approaches taken to determine how steroids affect the oviduct and to provide evidence for a possible role of the oviduct in embryonic survival. The first question asked was whether a nutritionally-induced decrease in progesterone concentrations in the first few days of pregnancy, related to an increase in embryonic loss, was due to an increased metabolic clearance rate of progesterone by the liver, or a decreased production of progesterone by the ovary. However, as described in Chapter 2, the nutritional regimen used did not create a difference in progesterone concentrations in the peripheral circulation and we were not able to test our main hypothesis. However, we did find that progesterone was already high in the oviductal circulation at a time when progesterone concentrations were rising in the peripheral circulation. This suggested that the oviduct would be affected by changes in ovarian steroids even before ovulation, and that changes in the timing of events in the oviduct could contribute to asynchrony and lead to embryonic loss. Those results led to two further studies looking at the effect of steroids on the oviduct environment.

The first study (Chapter 3) investigated temporal patterns of certain oviduct fluid components with respect to peripheral progesterone and estradiol-17ß concentrations through the peri-ovulatory period. Oviduct fluid components, total protein concentration, and oviduct-synthesized proteins, as well as peripheral steroid concentrations, were different in the pre-ovulatory period compared to the post-ovulatory period, establishing that the oviduct environment changes in this discrete window of time. Also, examining the oviduct environment in relation to ovulation is important to elucidating how the oviduct environment changes in this window of time, as the time of ovulation is variable in relation to onset of estrus in gilts (Almeida et al., 2000b). In the study described in Chapter 4, we used unilaterally ovariectomized gilts to establish the role of the sub-ovarian counter-current system, by creating differences in oviductal plasma steroids between oviducts of the same animal. We found differences in total protein concentrations and in secreted proteins in oviduct flushings, which were related to the differences in progesterone and estradiol-17ß in the oviductal circulation. This provided evidence for the functional importance of the countercurrent multiplier in local regulation of the oviduct environment.

Having found evidence for steroid-dependent regulation of the oviduct environment and for dynamic changes in oviduct fluid components during the peri-ovulatory period, the next step was to determine if differences existed in the oviductal environment in models where nutrition affected embryonic survival. In the fourth study (Chapter 5), a different nutritional model was used, in which gilts were fed different patterns of feed intake during the estrous cycle which had previously been shown to result in differences in embryonic survival and progesterone concentrations in early pregnancy (Almeida et al., 2000a). Unfortunately, there were no treatment effects on progesterone concentrations in this study, and we were not able to determine if progesterone-mediated effects on the oviduct environment could be a factor in embryonic loss. However, examination of oviduct fluid components demonstrated relationships between hormones in oviductal plasma and oviduct fluid components that were previously not evident, but now apparent due to the narrow sampling time in this study. The same nutritional model was employed in a final collaborative study, except that one group of gilts was given insulin in an attempt to counteract the detrimental effects of feed restriction on embryonic survival. At the same time relative to ovulation, feed restriction differentially affected the oviduct environment, as compared to insulin treatment, demonstrating that the oviduct can be a factor in embryonic survival in the pig.

For the first time in the pig, the research presented in this thesis describes changes in the oviduct environment in relation to ovulation, and directly investigates the oviduct environment in response to nutritional manipulation that results in decreased embryonic survival. The five studies presented as sequential chapters are extended forms of papers submitted for publication. The final chapter of this thesis discusses the use of appropriate nutritional models to elucidate a role of the oviduct in embryonic survival, and future studies that could be used to further examine the effect of oviduct function on embryonic survival.

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CHAPTER 1

LITERATURE REVIEW

A FACTORS AFFECTING EMBRYONIC SURVIVAL

The mechanisms of embryonic survival in the pig still remain to be fully elucidated, yet embryonic survival is a substantial determinant of litter size, as 20 to 50 % of embryos are lost by d 25 of pregnancy in the pig (Pope and First, 1985). The majority of the embryonic loss is thought to occur around the time of implantation, when blastocysts are rapidly elongating and start to synthesize estrogens. Embryonic diversity, and asynchrony of the uterus with the developing embryos, can result in embryonic loss (see Pope, 1988; Pope et al., 1990; Pope, 1994). Events that occur during pre-ovulatory follicular development will affect oocyte quality and steroidogenesis and may also contribute to embryonic loss. Independent of follicular development, other mechanisms that reduce peripheral progesterone concentrations in early pregnancy can affect steroid priming of the uterus, and thus alter the pattern of uterine secretion, contributing to asynchrony.

The role of nutrition in reproduction in the pig and other species has been extensively reviewed (I'Anson et al., 1991). There is little doubt that nutrients and their metabolites affect metabolic hormones that determine reproductive capacity through central gonadotropic or local ovarian mechanisms. However, effective use of nutrition to manipulate embryonic loss in the pig has offered a means by which to study the mechanisms involved in embryonic loss during the peri-implantation period (see reviews by Ashworth, 1994; Foxcroft, 1997). In these models, nutrition affects embryonic survival by altering oocyte maturation and folliculogenesis, and by altering steroid-dependent timing of oviductal and uterine secretions. The role of the oviduct as a factor in embryonic survival will be addressed in detail in later sections of this review. This section introduces the interactions between follicular development and steroidogenesis, and their influence on subsequent embryonic survival.

A. 1 *Follicular heterogeneity and oocyte maturation in embryonic survival*

In early pregnancy, the majority of embryonic loss occurs around implantation (d 10 to 18 of pregnancy) in the pig (Pope, 1994). Although the mechanisms involved are not fully understood, the peak of embryonic loss is associated with the commencement of embryonic estrogen synthesis. At the time when the embryo is capable of synthesizing estrogen it rapidly starts to elongate to a length of 1000 mm (Geisert et al., 1982a), and estrogen is thought to be a major signal in the tissue remodeling process. If estrogen is administered to a d 10.5 pregnant gilt in advance of blastocyst estrogen synthesis, total embryonic loss occurs by d 23, in contrast to estrogen administration on d 12, which has no effect on pregnancy rates (Geisert et al., 1991b). Interestingly, this estrogen production may be a key factor in determining embryonic survival, as d 5 embryos transferred to a d 6 uterus failed to develop when exogenous estrogen was administered at d 11 of the recipient's cycle (Morgan et al., 1987). The exogenous estrogen, mimicking blastocyst estrogen synthesis, alters uterine secretions (Geisert et al., 1982b; Geisert et al., 1982c) and creates a hostile or unfavorable environment for lesser developed embryos. Young et al. (1987) showed that administration of exogenous estrogen to pregnant gilts stimulated a release of intraluminal calcium and acid phosphatase activity by the uterus, whereas Vallet et al. (1998) recently demonstrated that changes in uterine protein secretion occurred in advance of blastocyst estrogen synthesis.

At the time of implantation a diverse population of embryos is present in the uterus (Pope, 1988; Pusateri et al., 1990), and the initiation of changes in the uterine environment by more developed blastocysts is detrimental to the development of less mature embryos (Pope et al., 1990). Xie et al (1990b) reported that lesser developed embryos at d 12 contained less estradiol, less total protein, and less acid-phosphatase activity than their more developed counterparts. Also, they demonstrated that these lesser-developed embryos were not able to stimulate uterine secretion to the same extent as more developed embryos. The mechanism responsible for embryonic loss at this stage may be due to the amount of total protein in the embryo. The lesser-advanced embryos have lower amounts of retinol-binding protein and may not be able to protect themselves from the increase in uterine secretion of retinol (Roberts et al., 1993). It therefore seems plausible that if the embryonic population within a litter is more uniform, then the embryonic survival rate can be improved (reviewed by Pope, 1990). In support of this, Blair et al. (1994) showed that sows with higher embryonic survival had decreased embryonic diversity at d 11. The highly prolific Meishan pig has an increased embryonic survival rate, and Bazer et al. (1988) suggested that the embryos in this breed are more uniform compared to commercial white breeds. However, Wilmut et al. (1992) and Anderson et al. (1993) later reported that the embryonic population can be as diverse in the Meishan as commercial white pigs. It is currently thought that the mechanism of increased embryonic survival in the Meishan is due to a slower developmental rate in Meishan embryos resulting in more gradual changes in the uterine environment (Youngs et al., 1993; Anderson et al., 1993; Youngs et al., 1994). This creates a less hostile environment for the less developed embryos and increases their chances of survival. However, Vallet et al. (1998) recently showed that the uterine secretion of proteins, although at a slower rate in Meishans compared to commercial breeds, was not influenced by the embryo, but by maternal progesterone secretion.

It seems that the establishment of some hierarchy of follicles is inevitable, as the more mature follicles can exert negative effects directly on neighboring follicles and indirectly control gonadotrophin secretion by the pituitary. Both Foxcroft et al. (1987) and Grant et al. (1989) showed a range of follicular maturational stages in the presumptive preovulatory follicular pool. Also, there is evidence that the maturity of follicular fluid bathing the oocyte is linked to increased nuclear maturation (Ding and Foxcroft 1994). Therefore, follicular heterogeneity may result in differential oocyte maturation, and subsequently in embryonic diversity. The embryos themselves may be genetically programmed to develop at different rates, which could be a function of how mature the oocyte is at ovulation and also the order in which the ova are shed (Pope, 1994). Evidence for this hypothesis was presented in a series of experiments by Xie et al. (1990a), in which they showed that the skewness of oocyte development continued into the early embryo population. They also demonstrated through fluorescence staining of oocytes that the oocytes destined to ovulate later gave rise to smaller blastocysts at d 12 of pregnancy (Xie et al., 1990b). Taken together the data implies that embryonic diversity originates with follicular heterogeneity, as Soede (1992) confirmed that embryo diversity is not related to duration of ovulation. In comparisons to white commercial breeds, the follicular population in the prolific Meishan is just as heterogeneous and ironically the follicles are smaller (Biggs et al., 1993). However, further studies by Hunter et al. (1993) and Faillace and Hunter (1994) demonstrated that the follicles of the Meishan, although smaller, were more estrogenic, more mature, and a greater proportion of the oocytes within the follicles had reached Metaphase II at ovulation. Futhermore, Xu et al. (1998) showed faster maturation of oocytes recovered from Meishan gilts when cultured in vitro with Meishan follicle shells, compared to culture of Large-White oocytes with Large-White follicle shells.

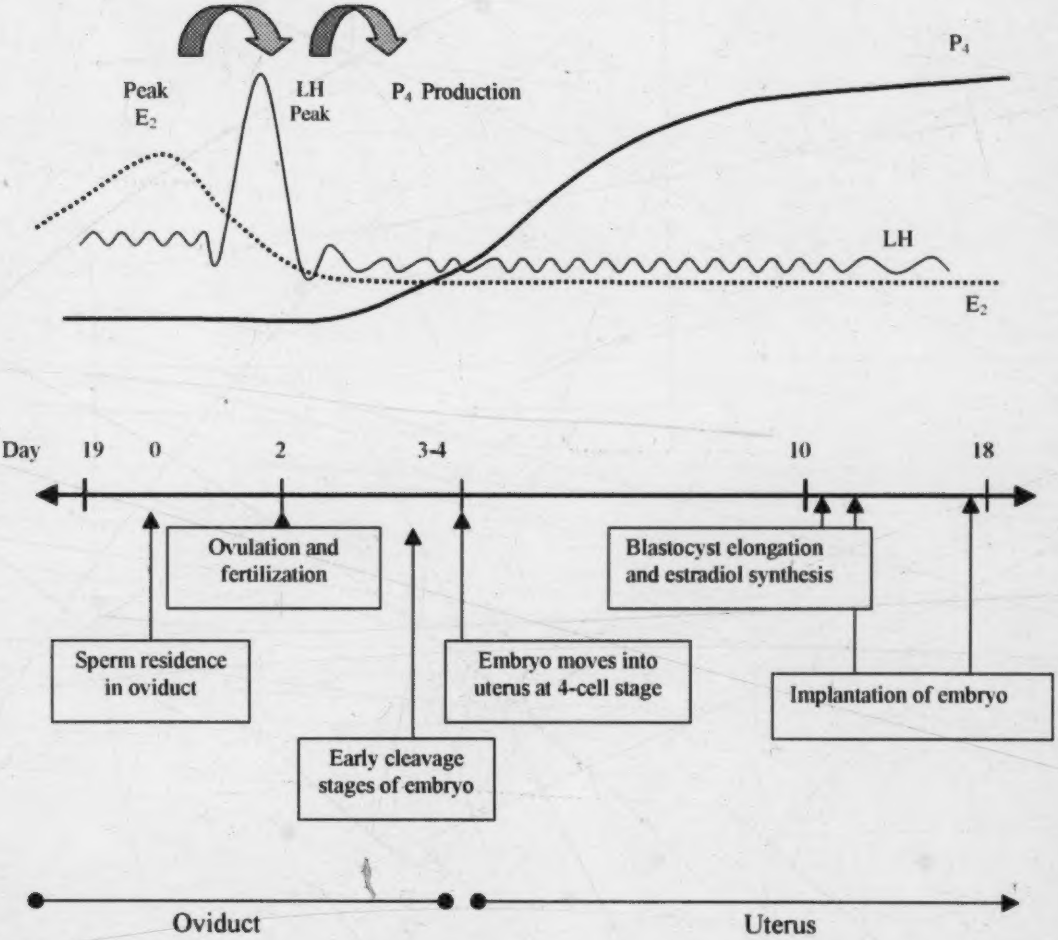
Alterations in oocyte maturation and folliculogenesis can be affected by restrict feeding during lactation in sows (Zak et al., 1997; Yang et al., 2000), as the estimated time for an antral follicle to mature to the preovulatory stage is 19 days (Morbeck et al., 1992). These changes in follicle and oocyte quality are possibly a mechanism by which restrict feeding can reduce embryonic survival in these models. Zak et al. (1997b) demonstrated that the extent of nuclear maturation was decreased in preovulatory oocytes recovered from sows that were more catabolic during late lactation (Zak et al., 1997a). In addition, the follicular fluid recovered from these sows was less able to support maturation of generic oocytes in vitro. There are two different mechanisms by which changes in follicular development may affect embryonic survival. The first is the steroidogenetic capability of the developing follicle, resulting in alterations in steroid priming of the maternal reproductive tract (covered in the next section), and associated effects on oocyte quality. If nutritional treatment causes the follicular population to become more heterogeneous, an increase in embryonic diversity may result in embryonic loss as previously discussed. However, the maturational stage of the oocyte at ovulation may have consequences for subsequent embryonic viability. McEvoy et al. (1995) reported that the manipulation of feed intake during the preovulatory period in ewes affected the in vitro developmental competence of embryos. In the pig, Graham et al. (1999) found that the proportion of embryos developing in vitro to the blastocyst stage from the 2 to 4-cell stage was higher in gilts fed ad libitum as opposed to restrict fed during the estrous cycle. Reduced pregnancy rates in cattle are associated with the use of a progesterone-releasing intravaginal device designed to synchronize estrus (Savio et al., 1993). Ahmad et al. (1995) subsequently demonstrated that the reduced fertility of these cows was probably due to altered oocyte maturation in cows with elevated estradiol concentrations due to a persistent dominant follicle. Therefore, oocyte maturity can affect embryonic development and viability, and heterogeneity in oocyte maturation has consequences in embryonic diversity.

A.2 *Steroid-dependent mechanisms of embryonic survival*

Consequences of a decrease in follicular maturity at ovulation are evident in steroidogenesis of the follicle, and are postulated to affect subsequent luteal function and embryonic survival (Foxcroft and Hunter, 1985; Hunter and Wiesak, 1990). If follicles are more mature at ovulation, it is then possible that luteal production of progesterone would be increased and result in a faster rise of progesterone in early pregnancy. The differences in estradiol and progesterone levels and the timing of their rise and fall during the periovulatory period may adversely affect embryonic survival in pigs (see Figure 1.1). Soede et al. (1994) has shown that a shorter interval between peak estradiol and rising progesterone concentrations is associated with higher embryonic survival. Also, Pharazyn et al. (1991a) and van den Brand et al. (2000) reported that higher progesterone concentrations in early pregnancy were also associated with increased embryonic survival in gilts and sows. Furthermore, a faster rise of progesterone concentrations after ovulation has been observed in the Meishan pig (Hunter et al., 1996), which, as a possible function of increased follicular maturity, may contribute to the higher embryonic survival observed in this breed. Nutritionally induced changes in embryonic survival are also associated with changes in progesterone concentrations in early pregnancy when feeding is restricted during pre-ovulatory follicular development (Zak et al., 1997a; Almeida et al., 2000).

Another mechanism that can decrease embryonic survival independent of follicular development and ovulation, is an alteration in the metabolic clearance rate of progesterone. This has been postulated in studies where a high plane of feeding after ovulation is associated with reduced progesterone concentrations and embryonic survival in pigs (Ashworth, 1991; Pharazyn, 1992; Jindal et al., 1996) and ewes (Ashworth et al., 1989). The nutritional effects are in part mediated by progesterone, as exogenous progesterone administration in early pregnancy can restore embryonic survival in pigs (Ashworth, 1991; Jindal et al., 1997) and ewes (Parr et al., 1987), and are independent of follicular development as treatments are imposed after ovulation. Both in ewes (Parr et al., 1993a; 1993b) and in pigs (Prime et al., 1993) a high plane of feeding results in an increase of metabolic clearance rate of progesterone by the liver. However, Freetly and Ferrell (1994) found that although splanchnic blood flow increased in pigs accustomed to a high level of feeding, the metabolic clearance of progesterone did not. Therefore, it may be that only acute changes in feed level are able to reduce progesterone concentrations by

Figure 1.1. Representative time-scale diagram of hormonal profiles and the events occurring during the peri-estrus period and early pregnancy in the pig.


Peak E_2
LH Peak
P_4 Production
P_4
LH
E_2
Day
19
0
2
3-4
10
18
Ovulation and fertilization
Sperm residence in oviduct
Early cleavage stages of embryo
Embryo moves into uterus at 4-cell stage
Blastocyst elongation and estradiol synthesis
Implantation of embryo
Oviduct
Uterus

this mechanism, and may partially explain the inconsistencies in available literature examining progesterone-mediated nutritional effects on embryonic survival (Dyck et al., 1980). Also, an alternative hypothesis is that peripheral progesterone does not mediate nutritional effects on embryonic loss, or is not affected in studies where increased feeding after mating did not affect embryonic survival rate (Dyck, 1991; Cassar et al., 1994).

Irrespective of which mechanism decreases peripheral progesterone concentrations in early pregnancy, effects on embryonic survival may be due to the steroid priming of the uterus. The uterus is only exposed to peripheral progesterone concentrations (Pharazyn et al., 1991b) and thus is sensitive to changes in progesterone from alterations in luteal production or metabolic clearance rate. Also, progesterone receptors disappear by d 10 of pregnancy in the uterus (Geisert et al., 1994), allowing the influence of embryonic estrogens to change the uterine environment in preparation for implantation after that time. Interestingly, induction of uterine protein secretion can be independent of the influence of the developing embryo (Vallet et al., 1998), and relies completely on maternal progesterone concentrations for appropriate priming. Therefore, a critical window of time is established between rising progesterone concentrations and d 10 of pregnancy when progesterone can exert its effects on the uterus. Secretion of proteins from the uterine epithelium is induced with progesterone (Knight et al., 1974; Roberts et al., 1976; Adams et al., 1981; Simmen et al., 1991), and the functions of these proteins has been extensively reviewed (Roberts and Bazer, 1988; Roberts et al., 1993). Examples of such proteins are uteroferrin and retinol binding proteins, which transport vitamins and nutrients to the developing embryo. Vallet et al. (1996) suggest that the increases in these proteins observed between d 10 to 13 may be dependent on the duration of uterine exposure to progesterone, as exogenous progesterone administered from day 3 of pregnancy accelerates the changes in these proteins (Vallet et al., 1998). This may be a critical mechanism in studies where timing of progesterone rise is altered.

Many other growth factors are secreted by the uterine epithelium, details of which are beyond the scope of this review. Some, such as IGF-I and IGF-II are progesterone induced (Simmen et al., 1990) and embryotrophic (Lighten et al., 1998). For reviews of these growth factors in early embryonic development, see Schultz and Heyner (1993), Simmen et al. (1993), Chegini (1996) and Kaye (1997). The contents of the uterine fluid are possibly critical for certain stages of development of the embryo (Corps et al., 1990; Keller et al., 1998), and if progesterone-induced protein secretion is altered, this may contribute to uterine asynchrony and lead to embryonic loss (see Pope, 1988; Pope, 1994). Results of transfer of embryos to asynchronous recipient uteri in the first week of pregnancy demonstrate how important appropriate uterine secretions are for the developing embryo. Geisert et al. (1991b) showed that embryonic survival was reduced when d 5 embryos were transferred to a d 6 uterus, and transfer to a d 7 uterus further augmented the effects on embryonic survival.

As previously discussed, the consequences of embryonic diversity become even more important if uterine secretion is asynchronous with the embryos. Ultimately, the uterus becomes receptive for implantation by the embryo, and this is thought to occur for a discrete window of time. The uterus also secretes proteinases and proteinase inhibitors (Menino et al., 1997; Jiang, 1999), and growth factors (Wollenhaupt et al., 1997; Gupta et al., 1998), in response to maternal steroids and embryonic factors to facilitate implantation. The coordinated communication between the embryo and uterus then becomes critical for successful implantation (Roberts et al., 1993). As a result, embryonic diversity, and inappropriate steroid priming of the uterus and resulting asynchronous development, together could exaggerate peri-implantation embryonic loss.

The oviduct has recently been implicated in the effects of nutrition on embryonic survival, although alterations in the oviduct environment have not yet been investigated. The oviduct can be affected by estradiol concentrations in the pre-ovulatory phase and progesterone concentrations immediately after ovulation due to the sub-ovarian countercurrent multiplier (Chapter 1, Section D). Therefore, the same mechanisms that affect follicular development in nutritional studies, may adversely affect the oviduct before ovulation due to alterations in steroidogenesis.

B OVIDUCT FUNCTION: GENERAL ASPECTS OF OVIDUCT PHYSIOLOGY AND OVIDUCT FLUID CHARACTERISTICS

B.1 *Basic Oviduct Morphology and Histology*

Gabriele Fallopius (1523-1562) first correctly described the oviduct, and termed it the "tuba uteri" after its resemblance to a brass musical instrument (Leese, 1988). Today, the terms 'tube' and 'uterine tube' are not as common, but the term 'oviduct' refers to the mammal, and 'Fallopian tube' is used mainly in the human. The oviduct is divided into four sections; the first is the pre-ampulla that surround the ovary to trap ovulated eggs by its fimbriae. The pre-ampulla includes the infundibulum and the pre-ampulla is, therefore, commonly termed the infundibulum or fimbria. Then, following the length of the oviduct toward the uterus, are the ampulla, isthmus and the uterotubal junction. The oviduct is composed of three major layers: an inner tunica mucosa, a middle tunica muscularis and an outer tunica serosa (Hook and Hafez, 1968). The mucosal layer is composed of epithelium and connective tissue, and the muscularis layer consists usually of an inner longitudinal layer and an outer circular layer of smooth muscle. The four segments vary considerably in their morphological, histochemical and biochemical characteristics, with differing degrees of muscularity, epithelial cell types and lumen structure. These differences were extensively studied with the light and electron microscopes, histochemistry and fixation techniques. Reinius (1970), using a fixation technique and an electron microscope, extensively described the morphology of the oviduct cells in the mouse in relation to gametes, embryos and oviduct secretion. His conclusions were that the pre-ampulla contains mainly ciliated cells and a few non-ciliated cells that have few or no signs of secretory activity. The ampulla contains many non-ciliated cells that seem to show proteinaceous secretion, whilst the isthmus contains two types of non-ciliated cells, those similar to ampulla and others that probably secrete a carbohydrate-rich secretion. The uterotubal junction consists of non-ciliated cells only. Without exception, the two types of cells present in the epithelium of the oviduct across mammals are the ciliated and non-ciliated (secretory) cells (Abe, 1996a). However, the proportional amount of these cells in regions of the oviduct across mammals is highly variable. In a review by Abe (1996a) species differences in the distribution of secretory and ciliated cells is discussed. For example, the isthmus contains mainly secretory cells across species, but in the ampulla, secretory cells predominate in the cow, whereas in the hamster, like the mouse (Reinius, 1970), ciliated cells are dominant. Such differences suggest variation in function of these regions of the oviduct across species.

Granules in the secretory cells can be distinguished by transmission electron microscopy as either electron-dense or electron-lucent. Dense granules are proteinaceous while less-dense granules are mucinous, and there are marked differences across species in the morphological features of these granules and their distribution in regions of the oviduct (Abe, 1996a). Degree of secretory activity and type of secretion may, therefore, be specific for a given region of the oviduct.

The degree of muscularity also varies across sections of the oviduct. The pre-ampulla consists of only two to three layers of muscle cells, mostly arranged longitudinally, whereas the isthmus consists of mainly circular muscularis, and the uterotubal junction is even more muscular in the mouse (Reinius, 1970). In the pig, the isthmic musculature consists of a thick, well-developed, circular inner layer and a thinner longitudinal outer coat (Hook and Hafez, 1968). There are species differences as to whether the circular muscle in the isthmus is the inner or outer layer. The uterotubal junction consists of both an inner longitudinal layer and an outer circular layer of muscle in the cow, sheep, monkey, rabbit and rat, whereas in the dog and pig, the inner longitudinal layer is missing (Hook and Hafez, 1968).

The contractions of oviduct muscles are under a combination of nervous and hormonal control (to be discussed in the next section). Response in spontaneous contractility of muscle to noradrenaline, and presence of noradrenaline in the isthmus, provides evidence of adrenergic innervation of the rabbit oviduct (Sachy et al., 1989). Also, there is peptidergic innervation of the pig oviduct, specifically somatostatin- and neuropeptide Y- containing nerve fibers (Happola et al., 1991). In addition, Czaja et al. (1996) using immunohistochemical techniques, found sympathetic noradrenergic fibers (tyrosine hydroxylase and dopamine-beta hydroxylase immunoreactive), neuropeptide-Y, somatostatin, vasoactive intestinal peptide, and substance-P related to vascular and smooth myocytes. Putative afferent fibers (Substance-P and/or calcitonin gene-related peptide immunoreactive), and parasympathetic efferent (vasoactive intestinal polypeptide or neuropeptide Y immunoreactive) fibers were located beneath the epithelium. These authors conclude that the distribution of these fibers implies their involvement in the regulation of blood flow and control of epithelial secretion.

The folds in the oviduct are also different across segments. The pre-ampulla has a large folds extending into the middle of the lumen, the ampulla has very small and sparse folds, the isthmus contains mainly circular folds, whilst the uterotubal junction lumen was almost closed in the mouse (Reinius, 1970).

B.2 *Cyclic changes in oviduct function*

The potential impact of the oviductal environment on embryonic survival is reinforced by the enhanced secretory activity of the oviduct during estrus (Buhi et al., 1989), at a time when gametes and embryos are present. Although hormonal control of synthesis and secretion of oviduct fluid components are discussed in later sections, the morphological characteristics of the oviductal epithelium, and oviductal contractions are also influenced by ovarian steroids. These basic aspects of oviduct physiology, and their differential state throughout the estrous cycle are critical for understanding oviduct function. During estrus, the oviduct transforms into a secretory organ and oviduct contractions are appropriate for transporting gametes, fertilization and transporting the early cleaving embryo to the uterus.

The distribution of secretory and ciliated cells in the ampulla changes through the estrous cycle, as an increase in the number of ciliated cells at estrus was demonstrated in the cow (Abe, 1996a). However, there were no cyclic changes in these cells reported for the hamster and rat (Abe, 1994; Abe, 1996a). The height of cilia in the preampulla and ampulla also changes throughout the estrous cycle in the pig and cow, reaching maximal height at estrus. In the cow, ewe, and pig, the number of cilia and ciliary rootlets varied on ciliated cells during the estrous cycle (Nayak and Wu, 1975). The cilia are primarily thought to bring ova to the fertilization site (Odor and Blandau, 1973). It was suggested that cyclic changes were evident in species with relatively long estrous cycles, but not in species like the rat and hamster with short cycles.

The pattern of muscular contractions in the isthmus also changes throughout the cycle in the pig. Rodriguez-Martinez and Einarsson (1982) dissected out the muscular layers of the pig oviduct to record spontaneous contractile activity and found that the frequency and force of both longitudinal and circular layers vary in the cycle. The circular layer demonstrated a hyperactive pattern around heat, but after ovulation a sustained wavy pattern was seen. In contrast, Borda et al. (1980) found no comparable differences in spontaneous contractile activity throughout the cycle. During estrus, the circular muscles moved in contraction waves (Rodriguez-Martinez and Einarsson, 1982), as also noted by Blandau and Gladdum-Rosse (1974) and originally proposed as the mechanism by which sperm were pushed to the fertilization site. In 1997, Muglia et al. used scanning electron microscopy to look at the three-dimensional muscular structure of the pig oviduct and concluded that it is likely more suitable for stirring rather than pushing gametes.

Borda et al. (1980) noted that there was a differential response to norepinephrine whether the oviduct tissue was pre- or post-ovulation. In pre-ovulatory tissue, norepinephrine suppressed motility of pre-ampulla and ampulla, but after ovulation stimulated motility in all tissues. The authors suggest that the changes observed in adrenergic responses of the sow oviduct may be linked to influences by ovulatory products. Battalia and Yanagimachi (1980) demonstrated that the combination of estrogen withdrawal and progesterone administration, not administration of estrogen or progesterone alone, triggered a propulsive movement towards the ovarian end in ovariectomized hamster oviducts. Also, in the rabbit, treatment with estrogen caused a decrease, whereas progesterone caused an increase in spontaneous smooth muscle contractions in vivo (Nozaki and Ito, 1987). These differences were also observed with steroid treatment in vitro, demonstrating a direct role for ovarian steroids in affecting contractility. In ovariectomized pigs, administration of progesterone, or estrogen and progesterone combined, induced an increase in noradrenaline concentration in oviduct tissue, demonstrating that steroids may affect the metabolism of noradrenaline in neurons supplying the porcine reproductive tract (Kaleczyc, 1994). ß2-adrenergic receptors were detected in bovine oviductal epithelium and increased expression of these receptors was observed with the addition of progesterone to oviduct cell culture (Einspanier et al., 1999). It is possible that ovarian steroids not only affect the catecholamine content of nerve fibers and alter contractions, but also through indirect and direct effects on the oviduct, to alter contractility or the oviductal response to neurotransmitters.

The oviduct epithelial cell ultrastructure changes drastically during estrus, as well developed secretory organelles, including secretory granules, are present (Murray, 1995), there are differences in the density of secretory granules (Hook and Hafez, 1968), and secretion rates are increased (Irritani et al., 1974). Hormonal control over the secretory activity of the oviduct is evidenced by studies done by Nayak et al. (1976). Three to four months after ovariectomy, epithelial cells were completely atrophied in the oviducts of gilts, but after just two days of estradiol treatment, secretory granules were present on the non-ciliated epithelial cells, and were maximally produced at three days of treatment. These secretory granules were arranged across the luminal epithelium at this time and it is assumed that their contents appear in the oviductal fluid. Estrogen and progesterone receptors are located in the oviduct throughout the estrous cycle, but during estrus in the gilt estrogen and progesterone receptors in the ampulla of the oviduct reach maximal concentrations (Stanchev et al., 1985).

Changes in secreted glycoproteins are even more finely tuned (Buhi et al., 1989; Buhi et al., 1990), and their glycosylation patterns (Raychoudhury et al., 1993) are even more specific throughout the estrous cycle, suggesting a specific role for steroid-induced gene expression and secretion of proteins. The role of the oviduct as an active secretory tissue is further established by the ability of individual oviduct epithelial cells, when placed on an adhesive surface, to organize themselves in to hollow tubes or spheres with microvilli directed toward the lumen (Joshi, 1995). The secretory activity of the oviduct is not modified by the presence of fertilized or unfertilized ova in the pig (Buhi et al., 1989), therefore it is the cyclic fluctuation of maternal steroids which contribute to the cyclic nature of oviduct function.

B.3 *Oviduct fluid properties*

The oviduct fluid is composed of many different ions, nutrients, proteins, growth factors and cytokines (see review by Menezo and Guerin, 1997). Some of these substances are derived from the serum and others are synthesized de novo in the oviduct epithelium; both types are selectively and actively secreted into the oviduct lumen to make up the fluid (see review by Leese, 1988). Other sources of oviduct fluid components are the peritoneal and follicular fluid. Using electrophoresis to observe protein patterns, Beier (1974) provided preliminary evidence that serum and the oviduct fluid are separate compartments, as several proteins are only present in the oviduct, not in serum. The highest rate of oviduct fluid formation (Wiseman et al., 1992), and protein synthesis (Buhi et al., 1989) is coincident with the estradiol peak immediately before ovulation in pigs, when gametes are present in the oviduct. The transformation of the oviduct into an active secretory organ at this time is suggestive of an active role of the oviduct to provide an optimal environment for fertilization and early embryonic development. Alterations in estradiol, and potentially progesterone concentrations, may adversely affect the oviductal environment and consequently, early embryonic development.

B.3.1 *Electrophoretic properties of the oviduct and fluid formation*

The mechanism and regulation of oviduct secretion has been poorly understood, as stated by Leese (1988). However, a few researchers have examined fluid formation indirectly through ion movement. The movement of fluid is secondary to the movement of ions, and therefore experimental designs were developed to test which ion(s) moved in a secretory fashion from the basal to apical side of the oviduct epithelium. These experiments were carried out after Leese and Gray (1985) developed an in vitro vascular perfusion technique of the oviduct.

The oviduct epithelium has been shown to secrete fluid coupled to secretion of chloride ions in the rabbit (Gott et al., 1988; Dickens and Leese, 1994) and in the human (Downing et al., 1997). Cl^- transport as a secretory ion was confirmed by demonstrating that blockage of Na^+-K^+-Cl^- co-transport and Cl^--HCO_3^- exchange affected chloride ion fluxes and oviductal fluid formation (Gott et al., 1988; Downing et al., 1997). Also, exogenous ATP is a potential modulator of oviduct fluid secretion, as inferred by its regulation of chloride secretion (Downing et al., 1997). A possible source of exogenous ATP is from sperm, which could explain a sharp increase immediately after mating in oviductal fluid formation in does (Gott at el., 1988).

However, Dickens and Leese (1994) using the same vascular perfusion technique in the rabbit oviduct, found that chloride ion flux did not change with a decrease in oviduct fluid formation. They suggested instead that potassium is an essential mediator because of the increase in fluid formation after administration of tetraethylammonium, which is a potent K+ channel inhibitor, concomitant with the action of Na^+-K^+-ATPase. This finding has not been repeated, and recent publications suggest that Cl^- ion flux is still generally accepted as the main mechanism of oviduct fluid formation (Downing et al., 1997). However, Dickens and Leese (1994) also reported that adrenergic agents could also regulate oviduct fluid formation, suggesting that these agents may be potent regulators of epithelial cell polarization. These findings also bring into consideration the fact that adrenergic receptors in pig vascular and muscular tissue (Czaja et al., 1996) are under ovarian steroid control (Kaleczyc, 1994). Since oviduct fluid volume exhibits cyclic changes (Bishop, 1956; Lippes et al. 1981; Sutton et al., 1984a; Wiseman et al., 1992), steroid-responsive adrenergic receptors may regulate the polarization of the oviduct epithelium for appropriate fluid formation rates during the cycle. Also, histamine has been shown to affect calcium and chloride ion movements in oviductal epithelial cells grown in a polarized layer as previously described (Downing et al., 1999), possibly adversely affecting oviductal fluid formation. These authors also reported that histamine affected oviductal smooth muscle contractions, providing another mechanism to influence oviduct function.

Bishop (1956) was the first to provide information on oviduct fluid formation and volume by ligation of the oviduct, and subsequent collection of fluid from rabbit does. During estrus, rabbit oviducts secrete 0.8 ml fluid/d, and this rate diminishes during pregnancy. He also demonstrated that exogenous estradiol administration restores the rate of fluid formation after ovariectomy. Lippes et al. (1981) collected human oviductal fluid and observed the highest volume, at 9.48 ml/d, during pre-ovulatory follicular development, coincident with the estrogen peak. In the pig, collection of fluid via catheterized oviducts revealed that oviduct fluid volume is highest at 1.18 ml/d during estrus, compared to 0.69 ml/d after estrus (Wiseman et al., 1992). Similarly, high oviduct fluid volumes were observed during estrus in the cow (Sutton et al., 1984a).

B.3.2 *Serum-derived factors*

Many ions, nutrients and amino acids are selectively transported into the oviduct, which implies that the selective transport exists to provide an optimal environment for gametes and embryos. For instance, oviduct fluid of rabbits has three times higher levels of potassium than plasma (Dickens and Leese, 1994) and the pH of pig oviduct fluid is between 7.5-8.0 due to bicarbonate ions present (Nichol et al., 1997a). There is not much evidence to suggest that the presence of serum-derived factors in the oviductal fluid are hormone-dependent, and therefore are potentially not involved in hormone-mediated changes in embryonic survival. However, they are included in this review to give the reader a sense of what oviductal fluid is composed of and its purpose. An understanding of the components of oviductal fluid provides information about the functional role of the oviduct in fertilization and early embryonic development. This information has also aided in vitro embryo culture, as embryos develop better in vitro when co-cultured with oviductal cells (see Section E), and provided insight into the specific requirements for embryonic development.

Serum is regularly used to support growth and viability of cells in vitro, yet it is clear that oviductal cell co-culture is advantageous to the developing embryo (Gandolfi and Moor, 1987). The ability of the oviduct to select certain serum proteins and secrete them in different concentrations to those seen in blood is important to embryonic viability. For instance, Walker et al. (1996) found a significant correlation with the mean number of nuclei per embryo and the addition of bovine serum albumin (BSA) to the culture medium, suggesting that BSA is embryotrophic. Although serum contains albumin, the concentrations of albumin and other proteins in the oviductal fluid are at 5-10% of the concentrations found in serum (Leese, 1988), and this may provide an optimal environment for the embryo. The most abundant of the serum-derived proteins are albumin and the immunoglobins, representing 60-80% of proteins in oviduct fluid (Menezo and Guerin, 1997). These proteins are selectively transported, because their concentration in the rabbit does not change with tubal fluid volume (Oliphant, 1978).

Synthetic oviductal fluid is routinely used for in vitro culture of embryos, which consists of serum-derived components at concentrations found in the oviduct. It is evident that this is beneficial to the embryo, as culture of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations, increased the proportion of embryos developing to the hatched blastocyst stage (Walker et al., 1996). Amino acid concentrations in oviduct fluid are higher when compared to serum, especially some amino acids that are thought to be utilized by or beneficial to the developing embryo (Menezo and Guerin, 1997). For example, glycine and alanine are present at higher concentrations than in serum, possibly because of their role in relieving the embryo of osmotic stress. Hypotaurine and taurine are also very high in oviduct fluid and may be involved in sperm capacitation. In addition, when hypotaurine is added to bovine embryo culture medium, blastocyst production and quality is improved (Guyader-Joly et al., 1998), indicating a role of this amino acid in embryo development. Amino acids present in oviduct fluid, and their functional roles are covered in detail by Menezo and Guerin (1997).

Using vascular perfusion, Leese and Gray (1985) demonstrated that glucose, pyruvate and lactate present in the oviduct lumen were serum-derived. The inability of unilateral ovariectomy to change the concentrations of these nutrients (Nichol et al., 1998), appears to confirm these findings and points to systemic control of these nutrients. Glucose concentrations in oviduct fluid are about 50% of the concentrations found in serum (Menezo and Guerin, 1997), yet lactate concentration is higher in oviduct fluid than serum (Leese, 1988). Higher lactate concentrations are due to the conversion of oviductal glucose to lactate (Leese and Gray, 1985), and may be important because lactate and pyruvate, not glucose, are utilized by the early embryo (Conaghan et al., 1993). The presence of these nutrients in oviduct fluid and their putative roles has been extensively reviewed by Leese (1988).

B.3.3 *Oviduct protein synthesis and secretion*

Oviduct protein synthesis increases during estrus and is associated with increased plasma estrogen concentrations (Buhi et al., 1990). Although, some proteins are progesterone-induced (see Section E.4, this Chapter), in general the oviduct-synthesized proteins are actively synthesized and secreted during estrus. The oviduct is capable of the synthesis and secretion of distinct proteins, some of which are present in other organs, such as growth factors and cytokines. However, there is a distinct family of glycoproteins that is only synthesized and secreted in the oviduct. The amino acid sequences of these glycoproteins are conserved across species (Verhage et al., 1998), and their synthesis and secretion is steroid responsive. Their possible functional roles are discussed in Section E, but Table 1.1 and Table 1.2 outline their characteristics pertaining to size, site of synthesis, association with gametes and/or embryos, and steroid responsiveness.

Buhi et al. (1991) gave exogenous estradiol and/or progesterone to ovariectomized ewes and found that estrogen induced or amplified two major basic proteins of 90-92 kDa. Again, Buhi et al. (1992) determined that two proteins of 100 kDa, and one 75-85 kDa protein, were produced by the oviducts of ovariectomized pigs in response to estrogen. Although the administration of exogenous steroids is useful in determining which proteins are induced by them, these experiments do not represent the physiological conditions present in the oviductal circulation in vivo. For example, in the sheep model above, although the 90-92 kDa proteins were secreted continuously after five days of estrogen treatment, these proteins are only

Table 1.1. Characteristics of estrus-associated oviductal secretory proteins across species.

Species	Glycoprotein name(s)	Size	Region	Steroid/hormone induced	Association
Human	HuOGP	102 kDa	Ampulla and Isthmus O'Day-Bowman et al., 1995)	Midcycle (O'Day-Bowman et al., 1995) Late-follicular (Arias et al., 1994)	Sperm (Lippes and Wagh, 1989) Oocytes (Verhage et al., 1998)
Mouse	GP215	215 kDa	Ampulla (Kapur and Johnson, 1986)	Estrus (Kapur and Johnson, 1986)	Oocytes and embryos (Kapur and Johnson, 1988; Kim et al., 1996b)
Hamster	Oviductin, GHOGP	160-250 kDa	Ampulla	Estrogen (Komiya et al., 1996; Abe et al., 1998)	Oocytes (Robitaille et al., 1988; Abe and Oikawa, 1990; St. Jacques et al., 1992) Sperm (Boatman and Magnoni, 1995)
Cow	EAP, bEGP	90-95k Da	Ampulla>Isthmus (Wegner and Killian, 1992; Staros and Killian, 1998)	Present during estrus (Wegner and Killian, 1992) LH (Sun et al., 1997)	Oocytes (Staros and Killian, 1998) Embryos (Wegner and Killian, 1991; Boice et al., 1992) Sperm (King and Killian, 1994; Abe et al., 1995)
Sheep	OGP, SOP92, oEGP	90-92kDa	Ampulla (Murray, 1992; Murray, 1993)	Estrus (Sutton et al., 1984a; Gandolfi et al., 1989) Estrogen (Buhi et al., 1991; Murray, 1993)	Oocytes and embryos (Gandolfi et al., 1989; Gandolfi et al., 1991) Sperm (Sutton et al., 1984b)
Pig		335kDa	Isthmus (Buhi et al., 1992)	Estrus (Buhi et al., 1989) Estrogen (Buhi et al., 1992)	
	POSP	85 kDa, 100 kDa	Ampulla>Isthmus (Buhi et al., 1996)	Estrus (Buhi et al., 1989) Estrogen (Buhi et al., 1992; Buhi et al., 1996)	Oocytes and embryos (Buhi et al., 1993)

Table 1.2. Properties of an oviduct-synthesized glycoprotein across species.

Species	Protein name	Mol wt (kDa)	Core protein		Glycosylation sites*		Notes	References
			Mol wt	Amino acids	N-linked	O-linked		
Human		102	70.5	633	4	Few	Chitinase-like	Arias et al. (1994)
Hamster	HOGP	102	70.89	650	8	Few	Mucin	Suzuki et al. (1995)
Mouse	MOGP	215	76.52	700	3	24	Chitinase-like	Sendai et al. (1995)
Sheep	oEGP	92	57	519	2	Few		Marshall et al. (1996)
Cow	bOGP	95	57.68	519	1	Few		Sendai et al. (1994)
pig	POSP	85	57.97	527	3	few	Chitinase-like	Buhi et al. (1996)

*potential

present in vivo in oviductal fluid on d 1 of the estrous cycle. Also, the progesterone administration pattern was continuous over 11 days, which mimicked the luteal phase, but does not accurately represent the steroid profiles during estrus when rising progesterone in the oviductal circulation is evident before ovulation (Hunter et al., 1983). More importantly, decreasing estradiol and increasing progesterone concentrations define the steroid profiles at estrus, and the response may be due to the changing E:P ratio. The evidence demonstrating the finely tuned patterns of oviductal protein secretion in different regions of the oviduct lend support to the hypothesis that local ovarian steroids are influencing the oviduct directly via the anastamosis located below the ovary (see Section D).

Lutenizing hormone also has a direct effect on oviduct glycoprotein secretion. Receptors for LH are found in the oviduct (Derecka et al., 1995) and LH has been shown to enhance blastocyst development in vitro (Gliedt et al., 1996). Functional LH receptors have been localized to bovine oviductal epithelial cells, and culture of these cells with hCG results in a dose- and time-dependent increase in OGP (Sun et al., 1997). Futhermore, the increase in OGP by hCG is not due to an increased transcription rate, but an increase in the half-life of the transcript. Because of the suggested role of OGP in fertilization and development of the early embryo, higher LH levels present during the peri-ovulatory period may directly participate in early pregnancy events by increasing the synthesis of OGP. Dickens et al. (1996) demonstrated that addition of exogenous ATP to human oviductal cells in vitro increased glycoprotein synthesis, providing another mechanism by which protein synthesis is regulated (Downing et al, 1997).

Because changes in the oviductal environment are complex and dynamic during fertilization and early embryonic development, it is possible that asynchrony between the embryos and the oviductal environment may augment embryonic diversity apparent in the uterus at a later stage. This theory is supported by studies in which ovulation is prematurely achieved through hCG administration and as a result, embryos do not survive (Hancock and Buttle, 1968). The embryonic loss is speculated to be due to the inability of the oviduct to create the appropriate environment for the gametes and or embryos, because it has not been appropriately primed with ovarian steroids. Another problem arises when the oviduct environment is prematurely advanced, as with exogenous progesterone administration. There was an increased incidence of polyspermy when progesterone was given before ovulation (Day and Polge, 1968), stressing again the role of ovarian steroids in establishing and maintaining an appropriate oviductal environment.

C MECHANISMS OF ACTION OF STEROIDS

Estrogen and progesterone, among other hormones and steroids, regulate virtually every aspect of oviduct function, and thus an understanding of their mechanisms of action is important. Estrogen and progesterone bind to receptors located within the epithelial cells and initiate expression of proteins and other factors, which are actively secreted into the oviduct lumen. Their receptors can also be activated via non-ligand binding, and the presence and role of growth factors in augmenting this effect is under investigation. There is evidence that a transmembrane form of the steroid receptor exists, and may be responsible for rapid changes in intracellular ions in sperm membranes. An understanding of hormone action will aid in elucidating how steroids regulate oviduct function.

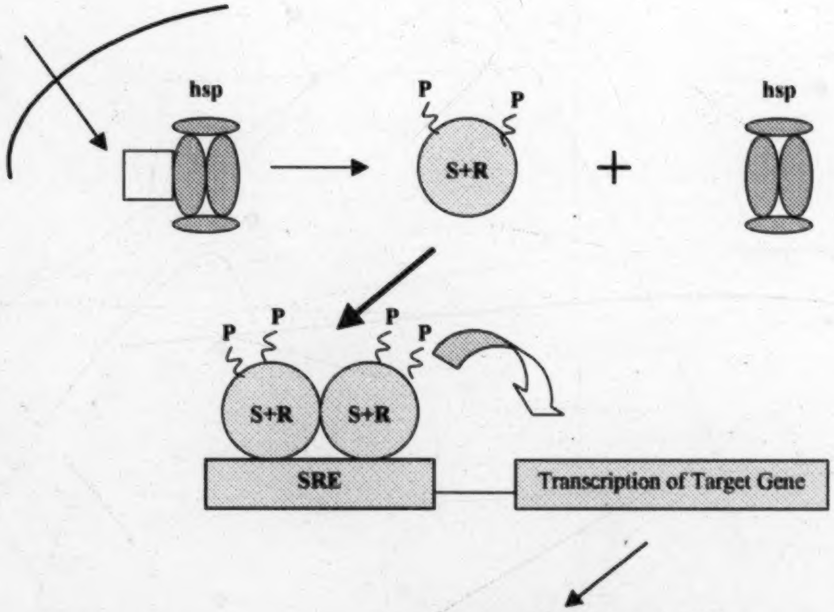
C.1 *Ligand –bound activation of gene transcription*

The estrogen and progesterone receptors are part of a large superfamily of ligand-activated transcription factors. They share a common structural and functional organization, with distinct domains that are responsible for ligand-binding, DNA-binding and transcriptional activation (Katzenellenbogen, 1996). The steroid diffuses into the cell and binds to its receptor, which changes its conformation and releases the receptor from an inhibitory complex containing heat shock proteins. The removal of the inhibitory complex exposes the DNA-binding site of the receptor. The steroid-receptor complex then forms homodimers, and binds to a promoter region of a gene that contains that specific steroid response element, initiating gene transcription (see Figure 1.2). The steroid-receptor complex acts as an enhancer, because it can interact with transcription factors and other components to modulate gene transcription. The consensus sequence of the estrogen response element is GGTCAnnnTGACC (Smith et al., 1995), and is found in the regulatory regions of steroid-responsive genes.

Steroid receptors are phosphoproteins, and the level of phosphorylation changes the function of the receptor complex, and hence modulates gene transcription. These levels of phosphorylation may be involved in ligand binding, receptor translocation to the nucleus, dimerization of the receptor-hormone complex, receptor binding to DNA and the receptor interaction with other proteins (reviewed by Blok et al., 1996). The progesterone receptor is phosphorylated under basal conditions, but when the ligand is bound, the steroid-receptor complex is hyperphosphorlyated, which then allows it to bind to DNA, where it is phosphorylated once more (reviewed by Blok et al., 1996)

When bound to its cognate ligand, the estrogen receptor phosphorylates rapidly, and the phosphorylation occurs on specific serine residues. Which residues get phosphorylated on the receptor seems to be cell-specific, as there is a discrepancy in the literature using different cell systems (Smith et al., 1995). Other factors can modulate the estrogen-induced gene expression via phosphorylation of the receptor. Protein kinase activators and cAMP are important, as they change the cell phosphorylation state and differentially phosphorylate the steroid receptor to modify and enhance transcription of certain proteins (see review by Katzenellenbogen, 1996) (see Figure 1.3). Also, serine 118 on the estrogen receptor can be phosphorylated via the mitogen-activated protein kinase (MAPK) pathway, thereby allowing growth factors that activate the RAS-MAPK cascade to activate steroid-induced gene expression (Blok et al., 1996). In addition, steroids and

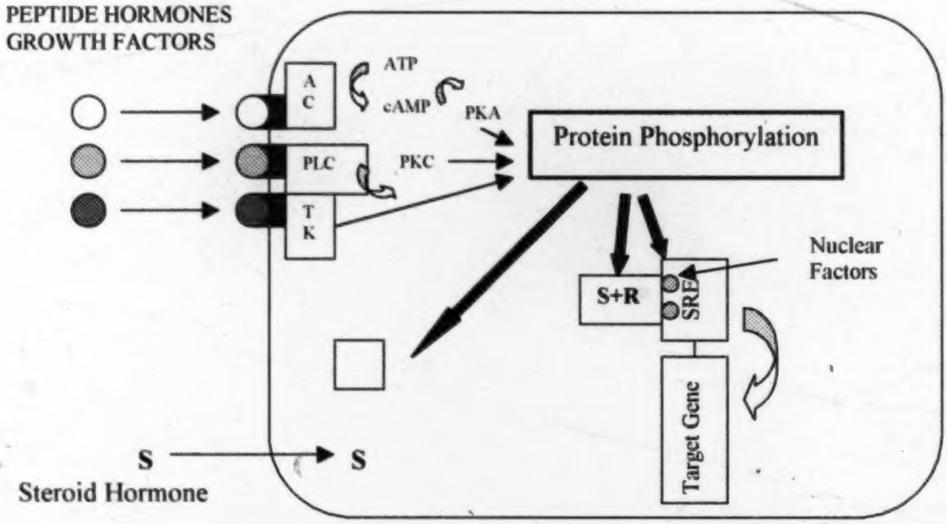
FIGURE 1.2. A model for steroid action on gene transcription. Steroids (**S**) diffuse across the plasma membrane of a target cell where they bind to receptors (**R**). The unbound receptors are associated with an inhibitory complex containing heat shock proteins (**hsp**). Steroid-receptor complexes are phosphorylated to varying levels depending on type of receptor, and presence of other regulators. Activated steroid-receptor complexes dimerize and act as transcription factors. They bind to the steroid response elements (**SRE**) of steroid-responsive genes present in the target cell and initiate mRNA synthesis. (Adapted from O'Malley et al., 1995; Spelsberg et al., 1996; Weigel, 1996)



Target gene synthesis can result in:

- Transcription of other genes (such as growth factors and receptors)
- Altered cell function (i.e. membrane properties, enzyme activity, RNA and protein processing)

Figure 1.3. Model for depicting protein kinase-steroid receptor transcriptional synergism. Phosphorylation via second messengers such as cAMP, and protein kinases –A (PKA), and –C (PKC), and through tyrosine kinase (TK) can occur when the receptor is not ligand bound, at the formation of the steroid-receptor complex, or when bound to the steroid response element (SRE) via phosphorylation of nuclear factors. The level of phosphorylation can alter the transcriptional activity as a result of steroid binding (adapted from Katzenellenbogen, 1996).


PEPTIDE HORMONES
GROWTH FACTORS
A
C
ATP
cAMP
PKA
PLC
PKC
T
K
Protein Phosphorylation
Nuclear
Factors
S+R
SRE
Target Gene
S
S
Steroid Hormone

oncoproteins also communicate through activator protein -1 (AP-1), which is a transcription factor of the fos/jun heterodimer, and functions to suppress steroid induced transcription (Schule et al., 1990), through mediation of the protein-kinase-C pathway (Angel et al., 1987).

An example of the complexity of this system is the up-regulation of the progesterone receptor by estrogen-receptor binding, due to phosphorylation of the estrogen receptor by signal transduction pathways initiated by insulin-like growth factor (IGF-I), epidermal growth factor (EGF) and cAMP (Aronica and Katzenellenbogen, 1991). Interestingly, Kraus et al. (1993) demonstrated that two promoters on the rat progesterone receptor gene exhibited differential responses to estradiol and to estrogen receptor-dependent stimulation by cAMP. They suggested that the functional differences between these two promoters might lead to altered expression of progesterone receptor isoforms, thereby influencing cellular responsiveness to progestins. Also, these two isoforms differ in their ability to suppress estrogen receptor-stimulated activity (Katzenellenbogen, 1996), further demonstrating the complexity of steroid-induced gene transcription, and the cross talk that occurs between steroids, their receptors and other factors in the cell.

C.2 *Non-ligand bound activation of steroid receptor*

In cell nuclei in the uteri of ovariectomized and adrenalectomized pigs, activated dimer receptor was present without estrogen binding (Jungblut et al., 1978), suggesting that in the absence of the steroid binding to its receptor, the receptor itself can still be activated to initiate gene expression. This can either be done as the receptor is attached to the response element, or by modifying the receptor before it is DNA-bound. The estrogen receptor is one in which the level of phosphorylation is related to its function (Blok et al., 1996).

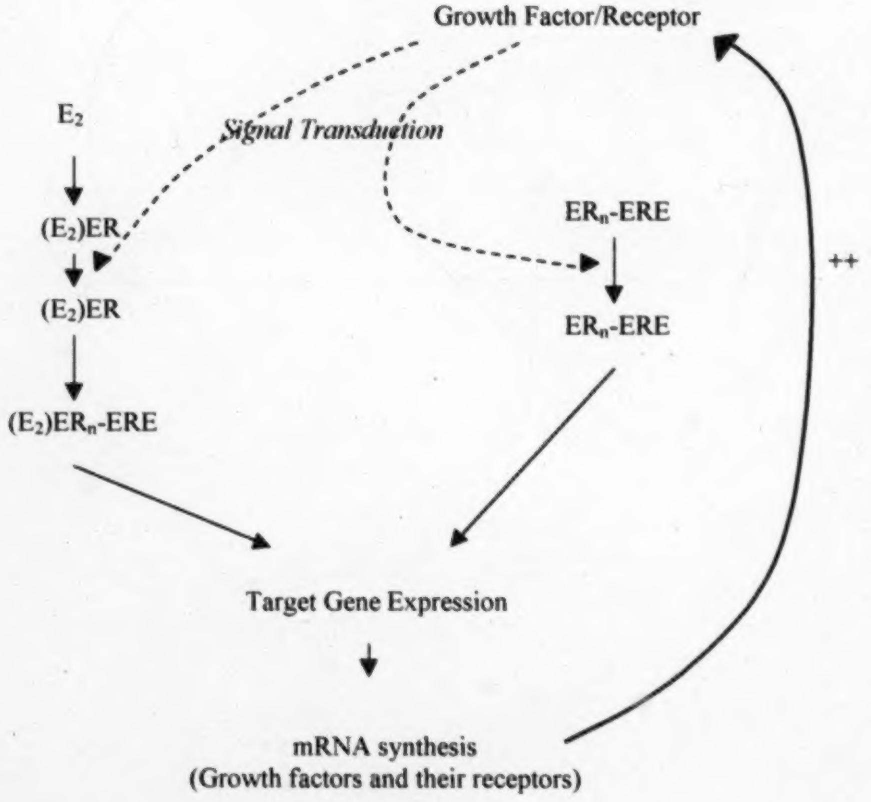
Peptide growth factors, especially EGF and IGF-I, are able to initiate transcription in the absence of estrogen binding, as they can modulate cellular phosphorylation pathways, as reviewed by Smith et al. (1995) (see Figure 1.4). It is possible that they have their own ligand-independent phosphorylation sites on the estrogen receptor, which helps explain estrogen-independent tumor growth. EGF, IGF-I, and cAMP, which all modulate steroid-induced gene expression by altering phosphorylation of the estrogen receptor, can potentially activate gene transcription through the receptor in absence of ligand in this manner. In addition, dopamine, via the signal transduction pathway activated through the dopamine type D1 receptor, is also able to turn on estrogen-dependent gene expression in a variety of cell types expressing D1 receptors (Smith et al., 1995).

The chick progesterone receptor can also be activated in the absence of steroid binding through modulators of protein kinases, such as growth factors and dopamine and alter phosphorylation of the receptor (reviewed by O'Malley et al., 1995); however, this is not the case in mammals (reviewed by Weigel, 1995). However, kinases and cAMP can modulate the activity of the progesterone receptor and modulate gene transcription and target cell function.

C.3 *Membrane-bound steroid receptor*

There are specific examples of responses to steroids acting at binding sites in membranes, for example the calcium influxes in sperm and hepatocytes induced by progesterone. These ion exchanges are rapid, non-genomic, effects of steroids and the mechanisms involved are different from the "classic" genomic theory of transcription activation. Recently, some steroid-binding proteins have been found in membranes of several types of cells. A progesterone membrane-binding protein has been characterized in porcine vascular smooth muscle by Falkenstein et al. (1996) which represents the first sequence information available for a steroid membrane receptor. Meyer et al. (1996) also characterized two membrane-binding sites for progesterone on porcine liver membranes. They are 28 and 56 kDa in size and could either be dimeric proteins or strongly associated. Progesterone binding to a

Figure 1.4. Model for ligand-dependent and ligand-independent activation of estrogen receptors in female reproductive tissues. Growth factors binding to membrane receptors activate signal transduction pathways that can phosphorylate the estrogen receptor (ER) in the presence of estradiol (E_2) and activate target gene expression. Growth factors can activate ER-dependent pathways when the ER (ERn) is bound to the estrogen response element (ERE), independent of E_2 binding. As a result, the target gene can initiate synthesis of growth factors and their receptors, causing the system to be self-perpetuating (adapted from Smith et al., 1995).


Growth Factor/Receptor
E_2
Signal Transduction
$(E_2)ER$
$(E_2)ER$
$(E_2)ER_n$-ERE
ER_n-ERE
ER_n-ERE
++
Target Gene Expression
mRNA synthesis
(Growth factors and their receptors)

progesterone receptor on the sperm membrane has been shown to mediate the acrosome reaction on stallion sperm (Cheng et al., 1998), and the mechanism by which this steroid membrane receptor elicits a cellular response is through rapid, non-genomic, steroid signaling (Falkenstein et al, 1999).

These membrane-binding sites may complement the genomic action of steroids at the nuclear level, and may be responsible for 'rapid' cellular changes that precede transcription. Also, whereas the accepted theory of steroid action suggests that steroids passively diffuse into the cell, the membrane-associated receptor may serve to attract steroids to that particular cell.

C.4 *Estrogen and progesterone receptors in the oviduct*

The ability of the oviduct to be stimulated by steroids resides in the presence and localization of steroid receptors and steroid response elements located on genes encoding other proteins. The oviduct can respond to ovarian steroids with contractility, cellular growth and differentiation, and protein synthesis and secretion. Buhi et al. (1992) suggest there is a differential response of ampulla and isthmus in the pig to ovarian steroids. In the pig isthmus, the cytosolic forms of both the progesterone and estrogen receptor do not vary, yet in the ampulla both receptors increase in concentration during proestrus and reach a maximum at standing heat, then decrease thereafter (Stanchev et al., 1985). The nuclear form of the estrogen receptor increases in both ampulla and isthmus during estrus and the early luteal phase, while the nuclear form of the progesterone receptor in both segments of the oviduct increases from estrus onwards (Stanchev et al., 1985). The ability of the receptors to be independently regulated by their ligand in different regions of the oviduct is indicative of segment-specific roles around fertilization.

Recently, work done by Rothschild et al. (1996) shows that an allele at the estrogen receptor locus is associated with a major gene influencing litter size in the Chinese Meishan pig. 50% of Meishans are homozygous for this allele, and Large

White breed pigs homozygous for this allele had litters higher by one piglet over Large Whites without the allele. Analysis of the estrogen receptor and the linked markers, suggested that the estrogen receptor was the best predictor of litter size differences. If this proves to be true, it would be interesting to study which genes are involved in the increase in litter size, and how the estrogen receptor is involved.

D THE SUB-OVARIAN COUNTERCURRENT SYSTEM: IMPORTANCE FOR OVIDUCT FUNCTION AND IMPLICATIONS FOR EMBRYONIC SURVIVAL

A counter-current exchange between the utero-ovarian vein and ovarian artery has been described for many species including the pig, (Ginther et al., 1972; Ginther and Del Campo, 1974; Ginther et al., 1973; Del Campo and Ginther, 1973) and allows for the direct transfer of a uterine luteolytic factor to the ovary (Ginther, 1974). This was confirmed by the transfer of radioactive PGF2α from the utero-ovarian vein to the ovary in sheep (McCracken et al., 1972) and pigs (Kotwica, 1980). However, there is also a countercurrent between the ovarian vein and the ovarian, oviductal and uterine arteries. This latter system allows local ovarian steroids to reach the adjacent oviduct to affect sperm, egg and embryo transport and the composition of oviduct fluid.

The first evidence for a sub-ovarian countercurrent system in the pig was provided by Krzymowski and co-workers in a series of experiments in which they infused [^{3}H]-labeled estradiol-17ß, progesterone and testosterone, or [^{51}Cr]-labeled red blood cells through a cannula into the ovarian vein in anesthetized sows (Krzymowski et al., 1981a; Krzymowski et al., 1981b; Krzymowski et al., 1982a; Krzymowski et al., 1982b). The experiments were controlled by surgically ligating the ovarian pedicle and ovarian circulation to isolate it from the systemic circulation. They collected the outflow from the ovarian artery and showed that when the radiolabelled steroids were infused, radioactivity appeared in the ovarian arterial blood. In a similar experimental design, Einer-Jensen and McCracken (1981) showed that progesterone was transferred in the ovarian vascular pedicle of sheep.

Evidence for the transfer of the ovarian steroids from the ovarian vein to the oviductal arteries was provided by Krzymowski et al. (1982c) using both in vitro (isolated ovaries and ovarian pedicles), and in vivo studies in sows. Hunter et al. (1983) further investigated the role of the countercurrent transfer mechanism in the regulation of oviduct function and sperm transport. The mechanism of transfer of steroids by this system was extensively reviewed by Einer-Jensen (1988) and Krzymowski et al. (1990). There is no evidence for vascular channels or connections between the ovarian artery and vein (Krzymowski et al., 1981b; Del Campo and Ginther, 1973). McCracken et al. (1984) found that the intact steroid was transferred, not a metabolite, and the ability of steroids to transfer from vein to artery is in part achieved by modifications to the structure of the epithelial membrane of the ovarian veins and arteries (Lee and O'Shea, 1975). McCracken et al. (1984) also showed that the greater solubility of the less polar steroids (progesterone versus 20α-dihydroxyprogesterone), enabled more efficient transfer and that this process occurs through a concentration gradient. In this study, only between 1-3% of steroids were transferred in a 90 minute period, but progesterone concentrations in the oviduct vein were reported to be at 10% of ovarian venous blood concentrations by Pharazyn et al. (1991b). The study by McCracken et al. (1984) was conducted with free, unbound steroids, yet it is assumed that only 1-2% of circulating steroid hormones are in the unbound form (Krzymowski et al., 1990). Krzymowski et al. (1982b) stated that the tissues surrounding the ovarian pedicle could retain up to 75-80% of the steroids draining the ovarian vein, so it is feasible that the transfer of these steroids is not simply concentration dependent. The lymphatic system above the anastamosis of the ovarian and uterine arteries functions in addition to the venous system for transport of steroids in the gilt from the uterus to the ovary (Magness and Ford, 1982). The lymphatics become greatly expanded at estrus (Leese, 1988), creating a larger lymph volume, and capacity for storage of steroids. Also, steroid concentrations were higher in the lymph and interstitial fluid than in venous blood in the sow (Kotwica et al., 1981). This pool in lymph may explain why ovarian pedicle and oviductal vein concentrations of ovarian steroids are not always related (Pharazyn et al., 1991b). Hunter et al. (1983) also noted that the levels of progesterone, estradiol and androstenedione corresponded in some but not all instances to the number of pre-ovulatory follicles or recent ovulations present on that ovary. It is evident that there is a complex system of steroid transfer in this countercurrent system, and there are many factors acting concurrently to regulate this. Current thinking is that the mechanism of transfer is through a concentration gradient, facilitated diffusion and active transfer of steroids through closely apposed veins and arteries, and also through interstitial fluid and lymph surrounding this network of veins and arteries.

The concentration of circulating gonadal steroids quantitatively and qualitatively affects oviductal fluids, both through a direct effect on epithelial cells and, indirectly, through their action on the vascular bed (Jansen, 1984). Both the concentration of estrogen, and the E:P ratio, in systemic blood are negatively correlated to the ovarian blood flow during the estrous cycle (Reynolds and Ford, 1984; Magness et al., 1983). In addition, α_1-adrenoceptors in the ovarian vascular pedicle, when blocked, increased the local concentration of androstenedione but not progesterone, and therefore changed the counter-current transfer of hormones (Stefanczyk-Krzymowska et al., 1997). Both stimulation and blocking of these receptors affected the concentrations of these hormones in the ovarian venous effluent. Therefore, nervous and hormonal regulation of ovarian blood flow can be an important component of the sub-ovarian countercurrent system, and affect the counter-current transfer of certain steroids.

D.1 *Influence of sub-ovarian countercurrent system on oviduct function*

The countercurrent system has been shown to increase ovarian steroid concentrations in the veins draining both the oviduct and uterus in ewes (Weems et al., 1989), but in the pig elevated concentrations of progesterone were limited to the oviductal vasculature (Pharazyn et al., 1991b). Hunter et al. (1983) also showed that progesterone, estradiol and androstenedione plasma concentrations were higher in the distal portion of the oviduct in pigs than in jugular veins during the pre-ovulatory period. The rise of progesterone in oviduct veins occurs in advance of ovulation due to the high concentrations of progesterone present in post-LH surge follicles (Grant et al, 1989). This rise of progesterone in the oviductal vasculature is well in advance of a peripheral rise (Pharazyn et al., 1991b), indicating that the local countercurrent mechanism may serve to prime the ipsilateral oviduct for upcoming events. This may be how ovulation products can alter sperm transport in the oviduct as proposed by Hunter et al. (1983).

The local effect of steroids on oviduct function is evident from studies that create a unilateral effect within an animal. In the study by Pharazyn et al. (1991b), a chance observation of a gilt bearing corpora lutea only on one ovary, demonstrated that elevated progesterone concentrations over peripheral levels were only observed in the side bearing the corpora lutea. This local effect has also been observed in cattle and sheep, whereby progesterone in the oviductal circulation opposite the ovary bearing the corpus luteum remains at peripheral levels, while the ipsilateral oviduct exhibits elevated levels of progesterone (Weems et al, 1989; Wijayagunawardane et al., 1996). Wijayagunawardane et al. (1998) examined six hormones and observed no differences in hormone concentration between the three parts of the oviduct, but found that progesterone, estradiol-17ß, PGE_2, $PGF_{2\alpha}$, and endothelin-1 were affected by whether the site of sampling was contralateral or ipsilateral to the dominant follicle or corpus luteum. They proposed that ovarian and oviductal products might synergistically control oviductal contraction for optimal embryo transport during the periovulatory period. The pH in oviducts during estrus in unilaterally ovariectomized gilts is not controlled as well in the oviduct ipsilateral to the ovariectomy as compared to the other oviduct (Nichol et al., 1997a), suggesting a local role for the ovary in maintaining a more constant and conducive environment for embryos. In contrast, unilateral ovariectomy in pigs did not affect oviduct fluid concentrations of glucose, lactate, and pyruvate, and the authors believe that these nutrients are regulated by systemic mechanisms (Nichol et al., 1998). An altered pattern of oviduct protein secretion was evident between ipsilateral and contralateral oviducts to a persistent dominant follicle (PDF) or a fresh dominant follicle in the cow (Binelli et al., 1999). The ovarian vein content of an ovary with a PDF is higher in estradiol-17ß than the opposite ovary (Ireland et al., 1994), demonstrating the potential effects of local steroids on oviduct function in this model. Binelli et al. (1999) suggest that the mechanism of altered oviduct protein secretion is through differential estradiol-17ß and progesterone receptor regulation, and the E:P ratio, on induction of proteins.

These local effects may alter oviduct function, embryo transport, embryo development and hence embryo survival. In regard to sperm transport, unilateral ovariectomy of rats resulted in almost complete suppression of sperm transport into the ampulla ipsilateral to the ovariectomy, an effect not seen on the ovary intact ampulla (Sultan and Bedford, 1996). Embryos recovered at d 6 of pregnancy from cows bearing a persistent dominant follicle were less able to reach the 16-cell stage than from cows ovulating a fresh dominant follicle (Ahmad et al., 1995). In vitro, bovine oviductal epithelial cells collected from an oviduct ipsilateral to an estrogen-dominant versus a progesterone-dominant cystic follicle supported an increased rate of development from a cleavage stage to hatched blastocyst stage embryo (Kamishita et al., 1999). Moreover, the co-culture of embryos with the oviduct cells ipsilateral to the estrogen-dominated cystic follicle also produced a significantly higher rate of development to the blastocyst stage. Also, conception rate at first service is also 37.1% versus 64.8% in heifers bearing PDFs (Savio et al., 1993), showing that the altered local oviductal and uterine environment from a PDF can affect fertility. The observation that normal embryos transferred to uteri at d 7 to cows bearing PDFs had the same pregnancy rates as controls (Wehrman et al., 1997), suggests that lower fertility resulting from a PDF is due to changes in oviductal not uterine environment. To date, no studies have examined embryo development in a unilateral ovariectomized model in the pig. Use of this model will provide considerable insight into the role of the oviduct in embryo survival, because the sub-ovarian countercurrent system affects only the oviduct, while the uterus is exposed to peripheral levels of steroids, in the pig.

E ROLE OF THE OVIDUCT IN GAMETE VIABILITY, FERTILIZATION AND EARLY EMBRYONIC DEVELOPMENT: IMPLICATIONS FOR THE ROLE OF THE OVIDUCT IN EMBRYONIC SURVIVAL

Early embryonic survival is largely determined by embryonic viability at the time of implantation. As described in Section A, two major factors affecting embryonic survival in a polytocous species are the variability of embryonic development and the synchrony of the embryos and the uterine environment. From the time of ovulation, the oocytes are transported through the ampulla region of the oviduct, are fertilized and undergo the first cleavage stages. The oviductal environment is thus very important in producing a viable embryo. This is further emphasized by the difficulty encountered with in vitro fertilization techniques and in culturing early embryos, especially in the pig. Oviductal cell co-culture has increased the ability of the embryo to become an expanded blastocyst in vitro in sheep (Gandolfi and Moor, 1987), and in humans, co-culture of fertilized human oocytes with hamster oviductal cells improved the implantation rate of embryos (Yeung et al., 1996). The oviduct is also an active secretory organ, and glycoproteins produced are reported to attach to the embryo and remain associated until d 7 of pregnancy (Buhi et al., 1993), suggesting a role of these oviductal proteins in early embryonic development. Oviductal secretion varies widely with day of the cycle and the fluid composition differs widely throughout the cycle. This suggests that the oviduct plays an active role in gamete maturation and transport, fertilization, and early embryonic development. Every aspect of oviductal function, such as muscular contractions (Nozaki and Ito, 1987), epithelial cell differentiation (Nayak et al., 1976), secretion (Irritani et al., 1974), and oviductal protein and growth factor synthesis (Buhi et al., 1997a), are under hormonal control. As discussed earlier, the oviduct is unique in that it is responsive to local concentrations of ovarian steroids and not the peripheral circulation. Therefore, is seems reasonable to suggest that embryonic loss that occurs later in pregnancy may very well be dictated by the events occurring during the embryo's residence in the oviduct.

E.1 *Oviductal influence on sperm transport and viability*

An important role of the oviduct is that it is the site of fertilization. The oviduct, therefore, is responsible for providing an appropriate number of viable sperm at the fertilization site, capable of fertilizing an ovum. The oviduct primarily acts as a selection and screening tool in determining which spermatozoa will be able to fertilize an egg, since only a few hundred sperm of the billions which are ejaculated reach the fertilization site at the ampullary-isthmic junction in the oviduct (see review by Hawk, 1983). Also, deposition of sperm into the reproductive tract of the female is in advance of ovulation, implying that the sperm are required to reside in the oviduct for a period of time in order to become capable of fertilizing ova. The spermatozoa become strongly associated with the oviductal epithelium, and are released at ovulation due to the influence of ovulatory products (see review by Hunter, 1995). There is evidence of modification of sperm membranes by the oviduct (Smith and Yanagimachi, 1991), alterations in protein synthesis of the oviductal epithelium with sperm attachment (Ellington et al., 1993), and co-culture of sperm with oviduct cells decreases polyspermic fertilization in vitro (Dubuc and Sirard, 1995). Sperm transport (Hunter et al., 1983) and secretion of specific sperm-binding oviductal proteins (King and Killian, 1994) are under the influence of ovarian steroids, and thus are potentially factors affecting fertilization rate and embryonic survival.

E.1.1 *Sperm transport in the oviduct and the function of the sperm reservoir*

In the past, sperm transport in the oviduct has been thought of as the coordination of smooth muscle contractions and cilia to aid movement of motile sperm to the site of fertilization. Blandau and Gaddum-Rosse (1974) removed oviducts from pigs and perfused them with fluid containing small particles and dye to track movement along the oviduct. They concluded that strong ciliary currents could transport particulate matter throughout the length of the isthmus to the ampullary-isthmic junction and that dye "boluses" moved like a chain of beads through the oviduct toward the ovarian end. Adding to this evidence was a study by Baker and Degen (1972), which demonstrated that both live and dead sperm were recovered from oviductal fluid of sows 4.5 to 15 minutes after insemination. This also confirmed the studies of First et al. (1968) that described the presence of boar spermatozoa in the ampullae of sows within 15 minutes after mating. The evidence points strongly to the contractility of the oviduct muscles in propelling the sperm to the fertilization site. Although this rapid phase of sperm transport is recognized, it is not the mechanism by which viable spermatozoa reach the fertilization site. As suggested by Blandau and Gaddum-Rosse (1974), this movement may serve to eliminate dead and non-fertilizing spermatozoa by transporting them into the peritoneal cavity. In rabbits, most of the sperm that reached the oviducts between 1 and 15 minutes after insemination were non-motile and had damaged membranes (Overstreet and Cooper, 1978). Therefore, evidence suggests that the sperm that are rapidly transported to the fertilization site after insemination, are not the population that fertilizes the ova.

It has become generally accepted in large mammals that large numbers of sperm are necessary to fertilize only a few ova. The final sperm: egg ratios at the fertilization site are critical, as a minimum number of sperm are needed to penetrate a single ovum, but if the number of spermatozoa at the fertilization site are artificially increased, polyspermy increases (Hunter, 1973). It is also important to note that there are other selective barriers of sperm transport to the fertilization site, such as the vagina, cervix and uterus, and their relative importance is dependent upon the species examined. For example, in species in which sperm are deposited into the vagina (e.g. horse, cow, sheep and human) the cervix would play a greater role in selection than in the pig, where sperm are deposited directly into the cervix or uterus. Therefore, especially in the pig, the oviduct functions to select and maintain viable spermatozoa in small numbers for proper fertilization of ova.

Estrus behavior in mammals is such that natural insemination occurs in advance of ovulation, suggesting that sperm is required to spend time in the oviduct to fully mature. In the pig, Hunter (1981) provided evidence that sperm need to spend a minimum of 1-2 hours in the oviduct in order to provide maximum fertilization, whereas in other species up to 8 hours are needed (Wilmut and Hunter, 1984). Spermatozoa gain the ability to fertilize ova during their residence in the oviduct, through the process of capacitation (Austin, 1951; Chang, 1951). Capacitation occurs in two phases; first, there is an alteration in the sperm membrane, followed by the hyperactivation of sperm to allow them to fertilize an ovum.

The uterotubal junction of rabbits, pigs, cattle, sheep, monkeys, rat and dog has projections into the lumen which form pockets resembling a sperm reservoir, in which sperm can be delayed or stored. Detailed information on the sperm reservoir and its function exists in a review by Suarez (1998). This sperm reservoir was thought to play a role in sperm capacitation (Hook and Hafez, 1968) and also to protect sperm from phagocytosis by immune cells of the female (Hawk, 1983). Smith and Yanagimachi (1990) flushed hamster oviducts 2 hours after mating, and reported that the vast majority of the sperm in the lumen were immotile, and those sperm bound to the mucosa or in crypts exhibited flagellar movement. The authors suggest that the sperm present in the lumen died shortly after their appearance in the oviduct, and therefore attachment to the epithelium served to protect the viable sperm. There is, however a limit to the length of time sperm can reside in the porcine reproductive tract without a decrease in the fertilization rate (Steverink et al., 1997). In their study, Steverink et al. (1997) inseminated sows at different intervals in relation to ovulation, with low (3 billion) and high (9 billion) sperm numbers. They concluded that the capacity of the sperm reservoir was very good when sows were inseminated between 0-12 hours prior to ovulation, even at low sperm doses. However, if insemination occurred 12-24 hours prior to ovulation or earlier, low sperm doses resulted in poorer fertilization rates, and no sows inseminated with 3 billion sperm had good fertilization rates. Insemination to ovulation interval was related to accessory sperm count, which demonstrates the ability of the sperm reservoir to regulate sperm viability until ovulation. In addition, Suarez et al. (1991) demonstrated that boar sperm attached to porcine oviductal explants in vitro maintained their viability after 24 h, although the numbers of sperm attached until that time had dropped significantly.

In vitro experiments have shown that only uncapacitated sperm can attach to the oviductal epithelium (DeMott et al., 1995; Fazeli et al., 1999). Since only freshly capacitated sperm are able to fertilize ova (Yanagimachi, 1994), it seems appropriate that if sperm become prematurely capacitated they are released from the sperm reservoir. Dubuc and Sirard (1995) found that only the spermatozoa that bound to oviductal cells in vitro were capable of fertilization, whereas unbound sperm produced a reduced penetration rate. Pollard et al. (1991) demonstrated that the fertilizing capacity of bovine sperm was maintained for 30 hours when sperm were incubated with oviductal cells, providing evidence that the oviduct maintains sperm viability. The attachment of the sperm plasma membrane to the oviductal epithelium is complex, suggesting that binding to the oviduct epithelium functions as a selection tool, to bind only viable sperm and maintain their viability until ovulation.

The complex binding of the sperm membrane to the oviductal epithelium involves carbohydrate residues (see review by Suarez, 1998). Terminal sialic acids have been found on the epithelium in the isthmus of rabbit (Menghi et al., 1995) and hamster (DeMott et al., 1995) and are possibly the sialic acid terminals of large glycoproteins on the surface of the epithelium. In both the pig (Raychoudhury et al., 1993), and the cow (Wegner and Killian, 1992), wheat germ agglutinin (recognizes terminal N-acetylgluocosamine resides) binding has been detected in the isthmus; however, Lefebvre et al. (1997) reported that sperm binding to the bovine epithelium involves fucose residue recognition. The observed differences in carbohydrate recognition across species make it apparent that the binding of sperm to the epithelium is species-specific.

As suggested by DeMott et al. (1995), these terminal carbohydrates are part of large glycoproteins on the surface of the oviductal epithelium. The oviduct secretes large mucin-like glycoproteins during estrus in the isthmus of the pig (Buhi et al., 1989), hamster (Paquette et al., 1995), and rat (Abe, 1996); which may be involved in coating the oviduct epithelium and promoting sperm binding. Suarez et al. (1991) noted that a mucous material that entrapped sperm was present on the epithelial surfaces of 23/32 isthmic and only 4/32 ampullar explants in the pig.

The release of sperm from their tight association with the oviduct epithelium is coincident with ovulation (Mburu et al., 1996). Although evidence has been presented to suggest that when sperm are capacitated, they are released from the oviductal epithelium irrespective of ovulation (see review by Suarez, 1998), this is most likely a coordinated process due to the dramatic change in ovarian steroid dominance from estrogen to progesterone concomitant with ovulation. Harper (1973) provided evidence with oviduct ligation that ovulatory products are stimulatory to sperm transport to the fertilization site in the rabbit. Hunter (1984) surgically ligated the oviduct 1.5 to 2.0 cm proximal to the uterotubal junction at various intervals approaching ovulation. He found that if ligation occurred up to 36 hours after mating, just prior to ovulation, fertilization of ova was essentially prevented. However, if ligation occurred immediately before, during or after ovulation, fertilization rates were 5%, 40% and 100%, respectively, demonstrating that the transport of viable spermatozoa was coincident with ovulation. Local ovarian steroids influence sperm transport via the local sub-ovarian counter-current system (Hunter et al., 1983), as unilateral ovariectomy results in virtually no sperm transport into the ampulla of the oviduct ipsilateral to the ovariectomy in the rat (Sultan and Bedford, 1996).

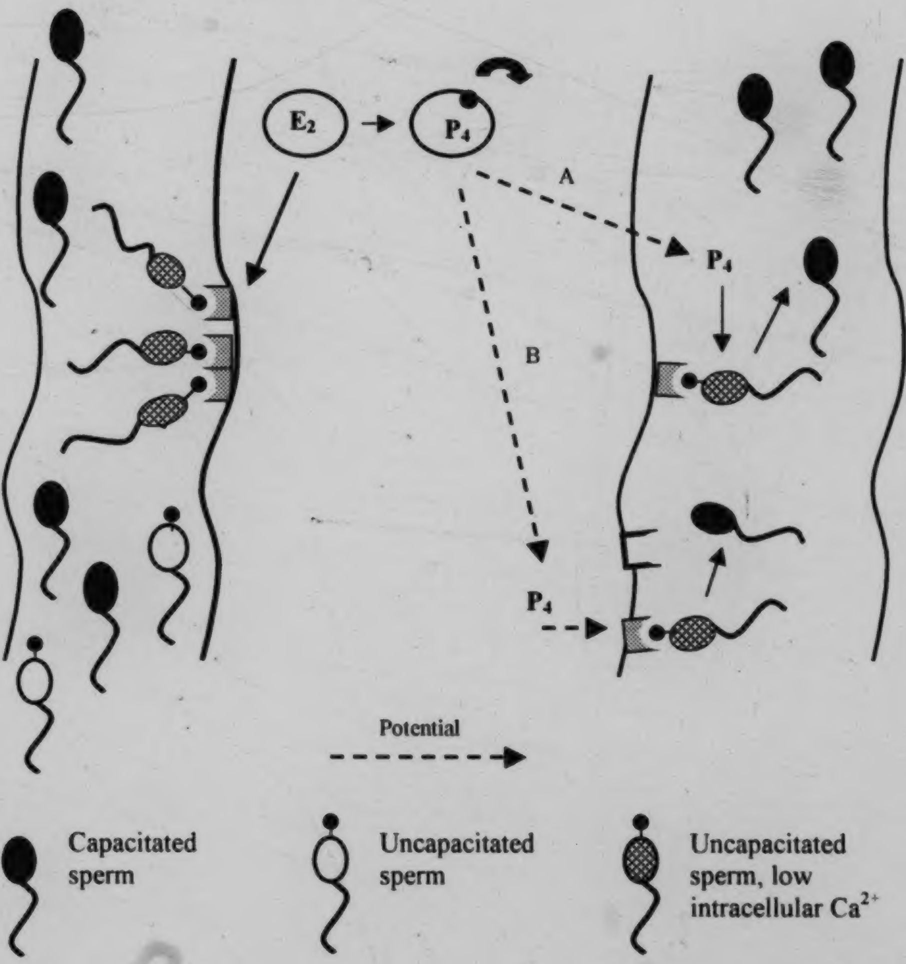
However, Hunter et al. (1999) tested the hypothesis that follicular fluid may leak into the oviduct at ovulation and by this mechanism, release the sperm from the epithelium. They added whole or steroid-free follicular fluid within 1 hour of ovulation into the isthmus of gilts, recovered the fertilized oocytes, and found that 34.8% of the oocytes were polyspermic in whole follicular fluid, as opposed to only 2.1% polyspermy with steroid-free follicular fluid. Therefore, although pre-ovulatory progesterone is suggested as the steroid influencing sperm release, follicular fluid is not the source of the progesterone. However, Hunter et al. (1999) proposed that progesterone affects the endosalpinx, by mobilizing calcium ions into bound spermatozoa. In contrast, Falkenstein et al. (1999) found that ligand binding of progesterone receptors on the sperm membrane were associated with a rapid influx of calcium ions. Also, Cheng et al. (1998) showed that binding of these membrane-bound progesterone receptors on the sperm surface was associated with the acrosome reaction. Taken together, these studies suggest that progesterone is directly involved with modification of the sperm membrane, causing their release from the oviductal epithelium. In support of this, Smith and Yanagimachi (1991) argue that release of the spermatozoa from the isthmic mucosa is due to a modification of the sperm surface, rather than a change in the mucosal surface.

Although it is coincident with ovulation, the exact mechanism of sperm release from the oviductal epithelium remains to be determined. However, it is most probable that the modifications in the oviductal epithelium due to increased progesterone concentrations in the oviductal circulation are related to the concurrent capacitation of sperm (Figure 1.5).

E.1.2 *Sperm capacitation and viability*

Capacitation is a biochemical process that changes the phospholipid content of the plasma membrane and thus enables the sperm to undergo the acrosome reaction. This is an absolute requirement for sperm passage through the zona, as the proteolytic activity of the acrosomal contents are required for dissolving the zona (Yanagimachi, 1994). Incubation of sperm with oviductal cells in vitro induces capacitation in human sperm (Yao et al., 1999), and boar sperm (Fazeli et al., 1999). The attachment of sperm to the isthmus is also important to capacitate sperm, as there was a greater proportion (45.6%) of fertilized eggs when sperm were deposited in the isthmus of gilts, rather than in the ampulla (1.4%) (Hunter et al., 1998). Also, oviductal co-incubation changes the sperm membrane's phosphatidylinositol content (Snider and Clegg, 1975) and capacitated sperm lose their ability to bind to sialic acid residues and wheat germ agglutinin (Mahmoud and Parrish, 1996), suggesting that modification of the sperm membrane is involved with capacitation. In addition, when equine spermatozoa are incubated with an antibody directed against plasma membrane components, their binding to oviductal epithelium in vitro is inhibited (Thomas et al., 1997), suggesting specific binding. Lefebvre and Suarez (1996) found that capacitation of sperm is involved with their release from the oviductal epithelium. Taken together, all of these data suggest that uncapacitated sperm bind to the oviductal epithelium, and when they undergo capacitation, a modification of the sperm membrane involves a loss of, or change in, the specific site(s) that bind the sperm to the epithelium.

Figure 1.5. Proposed action of sperm release in the oviduct. During estrus when estradiol (E2) concentrations are high, uncapacitated sperm become associated with the oviductal epithelium, functioning as a sperm reservoir. Already capacitated sperm do not attach and pass through the oviduct into the peritoneal cavity. Attachment to the oviductal epithelium maintains low intracellular Ca^{2+} in the sperm. Around ovulation, progesterone (P_4) may interact directly with progesterone receptors on the sperm head, raising intracellular Ca^{2+} in the sperm, causing their release from the epithelium (A). Also, P_4 may modify the oviductal epithelium, and cause sperm release (B).


E2
P4
A
P4
B
P4
Potential
Capacitated sperm
Uncapacitated sperm
Uncapacitated sperm, low intracellular Ca2+

Capacitation can occur spontaneously, but evidence suggests that the oviduct functions to delay capacitation through sperm binding to the epithelium in vitro (Murray and Smith, 1997). This is critical for fertilization, as capacitation allows the sperm to fertilize an ovum, but it also significantly reduces their lifespan (Yanagimachi, 1994). The mechanism by which attachment to the oviductal epithelium delays capacitation is through regulation of intracellular calcium in the sperm. Dobrinski et al. (1997) demonstrated that contact with oviductal epithelium maintained a low intracellular calcium ion concentration in equine spermatozoa in vitro. They also showed that the release of the spermatozoa from the oviductal epithelium was coincident with a higher intracellular calcium ion concentration. It is recognized that an influx of calcium ions is associated with capacitation (Singh et al., 1978), which demonstrates an overall link between sperm release and capacitation. However, it still remains to be determined how the epithelium maintains these low intracellular calcium ion concentrations, thus effectively preventing premature capacitation. This interaction is especially complex when one considers the fact that estrual oviduct fluid contains a capacitation factor (Parrish et al., 1989), suggesting that binding to the oviductal epithelium seems to protect sperm from this capacitation factor. However, Ellington et al. (1993) have shown that attachment of sperm cells to the bovine oviductal epithelium changes the types and quantities of proteins secreted in vitro, and therefore, as a result of the interaction, these new proteins may mediate the maintenance of sperm viability and delay capacitation.

Although studies suggest that there is no modification of the oviductal membrane during ovulation (Smith and Yanagimachi, 1991), it is more than just coincidence that the sperm are released and/or capacitated around ovulation. A very likely explanation is that an increase in progesterone concentrations immediately before ovulation may trigger mobilization of calcium ions into spermatozoa, as proposed by Hunter et al. (1999) and Falkenstein et al. (1999). The influx of calcium ions then causes the increased motility, or hyperactivation of spermatozoa.

Hyperactivation is an increase in flagellar beat amplitude and asymmetry that is observed in sperm recovered from the ampulla of the oviduct near the time of ovulation (see Suarez, 1998). So, although modifications to the sperm membrane cause dissociation between sperm and oviduct epithelium, the hyperactivity may enhance the ability of the sperm to release themselves (see reviews by Suarez, 1998; Smith, 1998).

The oviductal environment may also be important in maintaining viability of the sperm once it has detached from the epithelium. Human spermatozoa incubated with oviduct conditioned medium maintain their motility in vitro, due to a peptide secreted by the oviductal cells (Murray and Smith, 1997; Yao et al., 2000). This effect was also observed on boar sperm motility in porcine oviductal fluid in vitro (Nichol et al., 1997b). The ability of oviduct fluid to stimulate motility may also be due to the high concentrations of HCO_3^- present, as HCO_3^- has been shown to increase motility and mediate the acrosome reaction in hamster sperm (Boatman and Robbins, 1991). Ellington et al. (1998) have also shown that this increase in sperm motility by oviductal cell co-culture is not species specific, as human, macaque and bovine oviductal epithelial cells were all able to support sperm motility equally well. Interestingly, if bovine oviductal epithelial cells in vitro are treated with 17ß-estradiol or estrus cow serum, a higher percentage of spermatozoa are motile during co-culture as compared to control (no treatment) or pre-treatment of cells with luteal phase serum (Boquest and Summers, 1999).

E.1.3 *Sperm binding proteins*

Glycoproteins may be involved in the adherence of sperm to the sperm reservoir and capacitation, but they can also bind to the sperm membrane and possibly affect fertilization. Way et al. (1997), in various co-incubation scenarios with ampulla and isthmus fluid, found that bovine sperm binding to ova was improved if the sperm were first co-incubated with non-luteal phase isthmus fluid, suggesting a that factor from the oviduct adheres to the sperm membrane to enhance sperm binding to ova. Abe et al. (1995) found that the bovine oviduct specific glycoprotein (bOGP) bound to the posterior region of the head, the middle region and to the tail of the spermatozoa and could more effectively maintain viability and motility than BSA or gastric mucins. King and Killian (1994) reported that bovine estrus-associated glycoprotein (bEGP) bind so tightly with the sperm membrane that it could only be detected by immunoblot after the sperm membranes were permeabilized. The bovine (bEGP) in the study of King and Killian (1994) is 85-97 kDa in size and is probably the same protein as the 95 kDa bOGP, cited by Abe et al. (1995). In hamsters, Boatman and Magnoni (1995) also showed that the hamster oviductal glycoprotein bound to sperm membranes, and this glycoprotein was involved in sperm-oocyte binding. Thus far, only the bovine and hamster glycoproteins have been reported to be associated with sperm membranes, with the exception of observed sperm binding to the oviductal epithelium in the sperm reservoir in many species. Buhi et al. (1997) have found no evidence that the porcine oviductal glycoprotein (POSP) associated with sperm membranes.

Although, the role of these glycoproteins still remains to be elucidated, some possibilities have been proposed. Co-incubation of sperm with culture media containing semi-purified bOGP before in vitro fertilization did not improve fertilization rates (Martus et al., 1998), arguing against a functional role for bOGP in fertilization of ova. In contrast, the hamster glycoprotein is involved in sperm-egg binding, and improves the acrosome reaction (Boatman and Magnoni, 1995). Additionally, Hunter (1994) suggests that these glycoproteins may act to stabilize the acrosome, or prevent sperm agglutination. Another possibility is that by coating the sperm head and masking antigens, the glycoproteins may inhibit the production of anti-sperm antibodies and suppress an immune response.

E.1.4 *Oviduct-sperm interactions: Implications for fertility*

The above sections describe the interactions between the oviduct and sperm, with respect to sperm transport, capacitation, motility and viability. We also know that sperm release from the reservoir and the occurrence of capacitation (Hunter et al., 1983), and secretion of oviductal glycoproteins (see review by Jansen, 1995), are under hormonal control. Therefore, the principal influence of these events on fertility will most likely involve affects of fertilization rate, rather than embryonic quality. Ultimately, fertilization rate will be affected, reducing the number of embryos available for implantation. However, aberrations in normal hormonal profiles during estrus in the pig, caused by premature exogenous progesterone administration (Day and Polge, 1968), or deposition of excessive numbers of spermatozoa into the oviduct (Hunter, 1973), result in inappropriate sperm transport to the fertilization site. This affects the number of sperm available to fertilize ova, increasing the incidence of polyspermy, and thereby, early embryonic development. The complex interactions between the oviductal epithelium and spermatozoa thus provide a functional role for the oviduct in embryonic survival.

E.2 *Oviductal influence on oocyte maturation*

One of the biggest problems with in vitro fertilization is the occurrence of polyspermy. The co-culture of oviduct cells with oocytes for 3.5 hours before fertilization reduces polyspermy rates (Nagai and Moor, 1990) in gilts, suggesting a role of the oviduct in oocyte maturation. More important is the synchrony of the oviduct environment and ovulation, as a change from the estrogen-dominated environment of the oviduct in vivo to progesterone-dominated results in a 30% increase in polyspermy (Day and Polge, 1968). Proper cortical granule release is essential for a block to polyspermy in mammals, and the lack of proximity of cortical granules to the oolemma is seen in immature or aged oocytes in the pig

(Cran, 1989). The cortical granules release a protease that modifies a mouse zona pellucida protein (ZP2 to ZP2f) and results in the hardening of the zona (Ducibella et al., 1990). Kim et al. (1996a) found that complete cortical granule exocytosis occurred more often in vivo than in vitro in pigs, and demonstrated beneficial effects of incubation of oocytes with oviductal fluid in vitro. Although the addition of 1%(v/v) oviductal fluid reduced polyspermy rates and the mean number of sperm present in penetrated eggs, cortical granule exocytosis was not any better than in controls. However, when oocytes were cultured in 30% (v/v) oviductal fluid before insemination, the monospermy rate improved, without blocking sperm penetration, and cortical granule exocytosis was similar to in vivo conditions. Exposure of porcine oocytes to the oviduct environment improved their resistance to enzymatic digestion (Broermann et al., 1989), suggesting that a factor secreted by the oviduct may associate with or modify the zona pellucida. These results imply that a factor secreted by the oviduct is important in modifying the zona pellucida and for the complete maturation of cortical granules in oocytes before fertilization.

E.3 *Oviductal influence on fertilization*

If sperm and oocytes are mixed in vitro before they are placed in the oviducts, only 33% are penetrated (Baker and Polge, 1976), suggesting that the oviductal environment can enhance fertilization rates by acting on gametes separately. Subsequently, evidence to support a beneficial interaction of oviduct cells on sperm and oocytes used for fertilization in vitro was presented. Firstly, with respect to sperm, when human sperm are pre-treated with human oviductal cells in vitro, this promotes capacitation, stabilizes the acrosome, and suppresses sperm binding to the zona pellucida and fusion with the oocyte in vitro (Yao et al., 1999). Also, co-culture of boar spermatozoa with oviductal cells before in vitro fertilization reduced polyspermy rates (Dubuc and Sirard, 1995). Secondly, oocytes obtained from small and medium follicles and cultured with conditioned medium from oviducts in the pig exhibited a 95% improvement in normal fertilization rate and a 34 % decrease in polyspermy rate compared to oocytes treated with fetal calf serum (Vatzias and Hagen, 1999). Futhermore, this improvement was only seen with conditioned media obtained from oviducts of peri-ovulatory gilts, and not from oviducts obtained from luteal phase gilts, suggesting a specific steroid-induced oviductal environment that is beneficial to fertilization. The oviductal influence on gametes is also specific to the ampullary or isthmic secretions (Way et al., 1997). Even these may play different roles in fertilization because more bovine oocytes were fertilized if oocytes were cultured in ampullary fluid and sperm were cultured in isthmic fluid in vitro.

Gandolfi et al. (1991) stated that estrus-associated glycoproteins produced by sheep oviducts can bind to the zona pellucida of the oocyte or spermatozoa, implying a role in fertilization. Similarly, Staros and Killian (1998) found that bOGP was associated with zona pellucidae of bovine oocytes incubated with non-luteal phase fluid from the ampulla and isthmus. The application of these glycoproteins in the oviduct to the sperm and ova may be important for recognition binding during fertilization (see review by Nancarrow and Hill, 1995). Martus et al. (1998) compared the effects of bOGP and bovine serum albumin in fertilization medium and found that bOGP resulted in a higher fertilization rate (62.0% versus 31.2%), and the improvement in fertilization rate was eliminated by inclusion of an antibody against bOGP. They also showed by incubating gametes separately, that fertilization rates were improved when oocytes, not sperm, were cultured with bOGP before fertilization, and the addition of antibody against bOGP abolished this effect. This provides evidence of a specific interaction of bOGP with the oocyte and its possible participation in fertilization.

Other oviductal glycoproteins are known to bind to the zona pellucida of the oocyte in rodents. For example, an antigen called oviductin has been found to bind to hamster oocyte zona pellucidae (Robitaille et al., 1988; St. Jacques et al., 1992). The same oviductal glycoprotein in the hamster is also called ZP-0, because it binds to the zona pellucida and the perivitelline matrix and is thought to change the properties of the outer layer of the zona in reaction to testicular hyaluronidase (Abe and Oikawa, 1990). Furthermore, Boatman and Magnoni (1995) demonstrated that the hamster OGP enhanced sperm binding to the zona pellucida and improved the zona pellucida-induced acrosome reaction. This glycoprotein mRNA is only found in the ampulla (Komiya et al., 1996), which further substantiates the interaction of this glycoprotein and the oocyte. In the mouse, co-culture of oviductal explants with in vitro matured oocytes showed fluorescence staining within the perivitelline space, whereas the control oocytes did not (Kim et al., 1996b). In addition, the fluorescence staining was found in zona pellucida ghosts devoid of oocytes, demonstrating that there is a specific binding between the zona pellucida and this glycoprotein. Recently, a novel estrogen-regulated gene has been found in the rat oviduct epithelium through differential display techniques (Chen et al., 1999). This novel protein includes a domain common to zona pellucida sperm binding proteins, and may be important in fertilization. In the pig, POSP was localized throughout the zona pellucida and perivitelline space, and associated with the vitelline membrane of oocytes exposed to the oviductal environment (Buhi et al., 1993). Although a functional role for POSP binding in fertilization has not been investigated, it may function in sperm-ovum binding, or to protect the oocyte.

Although the sequence of these oviductal glycoproteins is highly conserved across species there is evidence to suggest that they function in species-specific sperm-ova binding (see review by Verhage et al., 1998). O'Day-Bowman et al. (1996) demonstrated that incubation of human spermatozoa and hemizonae with human oviduct-specific glycoprotein enhanced sperm binding to the hemizonae. Similarly, Schmidt et al. (1997) demonstrated that if ovarian hamster oocytes were inseminated with hamster sperm in the presence of hamster OGP, the number of sperm bound per oocyte was increased over the absence of hamster OGP. Also, hamster OGP addition had no effect on the number of sperm bound per oocyte when hamster oocytes were previously exposed to the oviductal environment before insemination. This study also demonstrated that the addition of human OGP to hamster ovarian oocytes decreased the number of sperm bound per oocyte and that pre-incubating human OGP with an antibody specific to it blocked this effect. Similarly, the addition of baboon and cow OGP during insemination of hamster oviductal oocytes also decreased the number of sperm bound per oocyte, indicating that homologous OGP enhances sperm binding to the zona pellucida, whereas heterologous OGP inhibits that effect.

Some insight can be gained by looking at the amphibian Xenopus laevis, as the role of the oviduct in modifying the oocyte is clearer. There is a protease with an M(r) of 66 kDa which is secreted by the oviduct and is 64% identical to serine proteases (Hardy and Hedrick, 1992) that processes a glycoprotein (gp43) on the egg envelope and alters its physiochemical and ultrastructural properties (Bakos et al., 1990). The egg envelope of the Xenopus is a coelomic envelope when ovulated, and after passage through the oviduct becomes a vitelline envelope. It is possible that oviductal proteins in mammals can adhere to and alter the oocyte in order to increase sperm binding or to mature the membrane so as to prevent polyspermy.

E.4 *Oviductal influence on early embryonic development*

The time an embryo spends in the oviduct is important for embryonic survival as demonstrated in a study by Chang (1950), whereby rabbit blastocysts transferred from the tube to the uterus prematurely, fail to implant. In co-culture experiments, the oviduct either secretes factors or absorbs embryotoxic metabolites which may improve the development of embryos past the species-associated block to the hatched blastocyst stage (Gandolfi and Moor, 1987) and improve implantation rate (Yeung et al., 1996). The developmental block in vitro seems to occur at a stage in each species corresponding to the activation of the zygote genome (see Bavister, 1988), which stresses the importance of the oviduct environment in facilitating genome activation.

Oviductal cell co-culture with embryos has been shown to improve early embryonic development in vitro in many species. Co-culture of human IVM/IVF derived embryos with human ampullary oviductal cells improves the proportion of embryos developing to the blastocyst stage (Hwu et al, 1998). Also, the percentage of viable bovine embryos after IVF is much higher when embryos are cultured with oviductal cells (Xu et al., 1992).

In general, embryos cultured in oviduct fluid and oviductal cells demonstrate improved development over embryos cultured in other media. However, there is evidence to show that blastocyst production is improved in co-culture with oviductal cells primed with certain steroid environments. For example, the cleavage rate of porcine embryos in vitro is significantly enhanced by co-culture with estradiol-treated oviductal epithelial cells (Xia et al., 1996b). Also, bovine blastocyst production from the cleavage stage (22.9% versus 37.9%) and 8-16 cell stage (39.5 versus 61.6%) is improved with oviductal cell co-culture from oviducts collected ipsilateral to a progesterone-dominated versus an estradiol-dominated cystic follicle (Kamishita et al., 1999). The authors indicate that the ability of the oviductal cells to facilitate embryonic development is closely associated with the steroid hormone profiles of the follicular fluid.

E.4.1 *Glycoproteins*

A 330 kDa glycoprotein is secreted specifically by the isthmus in the rat (Abe, 1996), and by the ampulla and isthmus in the pig (Buhi et al., 1989). This is a very large, mucin-like protein which appears to coat the embryo in the oviduct. It is heavily glycosylated with O-linked sugars and the hamster glycoprotein of corresponding size (160-240 kDa), is now classified as a secretory mucin (Paquette et al., 1995). It appears that species differences are mainly accounted for by the number of tandem repeats, or how many serine or threonine rich regions there are where O-glycosylation can occur. The rabbit oviduct also produces two different sialylated glycomolecules, one, which like the rat glycoprotein is secreted by non-ciliated secretory cells, and another with sialic acid-N-acetylgalactosamine terminal disaccharides (Menghi et al., 1995). This family of mucin-like glycoproteins may have a dual role in coating sperm to protect them and the developing embryo from the maternal immune system. These large glycoproteins have an extracellular association in the pig embryo and could be involved in cell-cell recognition (Buhi et al., 1993) or possibly play a role in establishing an appropriate nutritive environment for the developing embryo. Lastly, the mucin-like glycoproteins may function to coat the isthmus and protect the oviduct from implantation by the embryo. They commonly have sialic acid residues, which makes them 'slippery' and provides steric inhibition of binding. The lack of glycoprotein coating on human isthmus epithelium may explain why human embryos can implant ectopically (Jansen, 1995).

The smaller 90 kDa glycoproteins are present during estrus in oviduct fluid in the sheep (Sutton et al., 1984a; Gandolfi et al., 1989), pig (Buhi et al., 1989), cow (Boice et al., 1990; Wegner and Killian, 1992), and human (O'Day-Bowman et al., 1995). Gandolfi et al. (1991) localized the sOP92 glycoprotein to the zona pellucida, perivitelline space and on the blastomere membranes on 1 to 8-cell sheep embryos. Similarly, the pig oviductal glycoprotein, POSP, has also been immunolocalized to the zona, perivitelline membrane, and perivitelline space, and remains associated with the embryo until the hatched blastocyst stage (Buhi et al., 1993). Also, Boice et al. (1992) demonstrated using immunocytochemistry techniques that bovine embryos at the 4- and 8-cell stage, but not ovarian ova, had the oviduct-specific glycoproteins associated with their zona pellucidas, perivitelline space and membrane, and speculated that these proteins are biologically important to the developing embryo. Wallenhaupt et al. (1996) purified the 97 kDa glycoprotein in the pig, and using in vitro fertilized porcine oocytes cultured with this protein, showed an increase in the rate of incorporation of methionine into protein by 4-cell embryos. The glycoproteins could be acting to stabilize the physical environment surrounding the embryo, but more importantly they might be acting as carrier molecules to present cations and metabolic substrates at appropriate concentrations to the vitelline membrane (Hunter, 1994). The possible roles of these glycoproteins in early embryonic development have been the subject of reviews by Murray et al. (1995), Nancarrow and Hill (1995) and Buhi et al. (1997), and are summarized in Table 1.3.

E.4.2 *Growth factors, cytokines and other proteins*

The oviduct also synthesizes and secretes growth factors, cytokines and other proteins that may be embryotrophic (see Table 1.3). Evidence for the synthesis and secretion of many of these factors by the oviduct is accumulating rapidly. The growth factors may act in an autocrine or paracrine manner to regulate or modulate oviductal function (Gandolfi, 1995) or be important for embryonic and fetal growth (Schultz and Heyner, 1993). In the pig, the 4-cell stage is very long (Polge, 1982), and is thought to be due to the synthesis of new proteins to activate the zygote genome (Telford et al., 1994). Species differences with respect to expression of receptors and their ligand may be explained by species differences in embryonic genome initiation. The switch from maternal genome to activation of the zygote genome is early in the mouse, at the 2-cell stage, at the 4-cell stage in the pig, and at the 8-16 cell stage in the sheep and cow (see Bavister, 1988; Telford et al., 1994 for review). The timing of the activation of the embryonic genome in most species is also related to the movement from oviduct to uterus (Bavister, 1988). Growth factors in the oviduct fluid are thought to trigger the synthesis of new proteins in the zygote and also in the blastocyst.

Oviductal growth factors and proteins have been implicated in mitogenic and proliferative actions in the developing embryo during its residence in the uterus, in protection of the embryo, and providing an appropriate microenvironment for its development (see reviews by Schultz and Heyner, 1993; Kaye, 1997) (see Table 1.3 for summary). Although the oviduct is capable of synthesizing these factors, their appearance in oviduct fluid may also represent a serum transudate. The information

TABLE 1.3. Growth factors, proteins and cytokines synthesized by the oviduct, potentially involved in early embryonic development. Species is indicated in brackets; p=porcine, b=bovine, m=mouse, ham= hamster, h=human, o=ovine.

Item	Possible role in early embryonic development
Large (160 to 350 kDa) oviduct-specific glycoproteins	Protection of embryo from maternal immune system Establishing a micro-environment surrounding developing embryo (Buhi et al., 1997a) Improved sperm-egg binding in vitro (ham: Schmidt et al., 1997)
Smaller (80-100 kDa) oviduct-specific glycoproteins	Improved fertilization in vitro (b: Martus et al., 1998) Increased number of IVM/IVF embryos proceeding to the 4-cell and 16-cell stage (p: Buhi et al., 1997a) Increased protein synthesis in 4-cell embryos in vitro (p: Wallenhaupt et al., 1996) Act as carrier molecules for substrates needed by the embryo (Hunter et al., 1994)
IGF-I	Increases cell number in inner cell mass in vitro (m: Smith et al., 1993; h: Lighten et al., 1998) Inhibitory effects until blastocyst stage, then positive effects onwards in vitro (m: Shi et al., 1994) Increased embryonic development in vitro (b: Matsui et al., 1995; h: Lighten et al., 1998)
IGF-II	Stimulates protein synthesis in embryos in vitro (m: Shi et al., 1994; in Kaye , 1997) Stimulates cell proliferation and blastocyst formation in vitro (m: in Kaye, 1997) Increases rate of embryonic development (Rappolee et al., 1992)
IGFBP 1-6	Increased levels of IGFBP-3 are embryotrophic in vitro (h: Lai et al., 1996; o: Stevenson and Wathes, 1996)
EGF and TGF-α	EGF involved with inner cell mass proliferation and implantation (m: Sibilia and Wagner, 1995) EGF increases rate of embryonic development (m: Paria and Dey, 1990) Embryonic TGF-α acts on embryonic EGF receptors to facilitate embryonic development (m: Paria and Dey, 1990; Kaye, 1997) Oviductal EGF and TGF-α act to stimulate embryo development (m: Morishige et al., 1993)

Item	Possible role in early embryonic development
TGF-ß 1,2,3 and basic FGF	Involved with peri-implantation events, promoting synthesis of extracellular matrix factors (Schultz and Heyner, 1993) Oviductal expression of TGF-ß thought to regulate oviductal epithelium TGF-ß with bFGF increased proportion of blastocysts in vitro (Larson et al., 1992a) Basic FGF acts as a mitogen (Schultz and Heyner, 1993)
PDGF-α and -ß	Increases embryonic development in vitro, and anti-PDGF reduces the inner cell mass (b: Larson et al., 1992b; Thibodeaux et al., 1993)
CSF-1	Involved in implantation events (m: Arceci et al., 1989) Cellular proliferation and early embryonic development (p: Jiang, 1999)
LIF	Regulation of cellular proliferation in embryo (m: Schultz and Heyner, 1993) Possibly involved with tubal implantation in human (h: Keltz et al., 1996)
TIMP-1	Stimulates embryonic development in vitro (b: Satoh et al., 1994)
PAI-1	Possible protection of embryos from proteolytic damage in the lumen (p: Buhi et al., 1997a)

pertaining to the appearance of these factors in the oviduct, their regulation, and their interactions with the developing embryo is limited, yet the purpose of this review is to bring together information about these growth factors and to speculate on current hypotheses. Special attention will be given to the insulin-like growth factor family and their binding proteins, because these are the growth factors studied in the research presented in this thesis.

E.4.2.1 *Insulin-Like Growth Factor (IGF) Family*

The anabolic and mitogenic effects of the IGFs on many cell types are well known and are the subjects of recent reviews, one of the most extensive being that of Jones and Clemmons (1995). Recently, this family of growth factors and their binding proteins has been implicated in early embryonic growth and development (see reviews by Schultz and Heyner, 1993; Chegini, 1996; Heyner, 1997). Gene-knockout studies on transgenic mice demonstrated that deletion of the type I receptor, IGF-I or IGF-II resulted in the mice having smaller or non-viable pups (Baker et al., 1993; DeChiara et al., 1990), indicating that these genes are necessary for proper embryonic or fetal growth. Insulin and IGF-I are thought to play a role in growth promotion during early embryogenesis in species as diverse as sea urchins, Xenopus, and flies, as described in a review by Schultz and Heyner (1993).

Kaye (1997) in a review of preimplantation growth factor physiology illustrated two possible growth factor circuits for both the early embryo and the developing blastocyst. For both circuits, maternal insulin secreted by the pancreatic ß-cells is involved in endocrine regulation of preimplantation embryonic physiology. Insulin has been shown to stimulate cell proliferation in the inner cell mass (Harvey and Kaye, 1990) and to increase protein synthesis in the trophectoderm (Harvery and Kaye, 1988) in mouse embryos. Also, pig blastocysts in vitro respond to physiological levels of insulin through an increased rate of protein synthesis (Lewis et al., 1992). Insulin affects the embryo by binding to insulin receptors and also to the type I IGF-I receptor, however it is thought that its anabolic action on embryos is mainly through the type I receptor (Kaye, 1997). The stage of embryonic development when these receptors are expressed differs across species, which does not provide a clear role for insulin in early cleavage stage embryos. In the mouse, mRNA for the insulin and type I receptor was not detected until the late 8-cell to morula stage, and for the IGF-II receptor at the 2-cell stage (Rappolee et al., 1990; Schultz et al., 1990). Using immunofluorescence staining techniques, positive staining for insulin was not detected until the morulae stage (Rosenblum et al., 1986). In the cow however, all three receptors were detected at the one-cell stage (Winger et al., 1997), and we do not know how early these receptors are expressed in the porcine embryo. In addition, although many studies have looked at insulin's effects on embryos at the blastocyst stage, little is known about its actions on the cleavage stage embryo, at a time when it resides in the oviduct. Thus far, addition of insulin to bovine embryo culture increased the percent of embryos developing to morulae by d 5 in vitro (Matsui et al., 1995). Lastly, the effects of insulin are also mediated by the presence of insulin receptor substrate-1/ docking protein (IRS-1), which is the principal substrate for insulin and IGF-I receptor tyrosine kinases, as reviewed by Heyner (1997).

IGF-I, unlike insulin, can be produced by many cell types in addition to its primary production by the liver in response to GH (Dunaiski et al., 1999). The role of IGF-I in the uterus and oviduct is either through actions on the pre-implantation embryo or in the differentiation and proliferation of the epithelium. As with insulin, much of the data available on the effects of IGF-I on the developing embryo have been examined at the blastocyst stage. IGF-I, unlike insulin, selectively increases the number of cells in the inner cell mass (Smith et al., 1993; Lighten et al., 1998) and is associated with effects on cell proliferation in cleaving embryos and blastocysts, and differentiated actions in blastocysts (see review by Kaye, 1997). Lighten et al. (1998) demonstrated that supplementation with IGF-I into culture medium increased the proportion of human embryos developing to the blastocyst stage from 35% to 60%, and that this effect was mediated through the IGF-I receptor. Similarly, IGF-I addition to culture medium increased the number of bovine embryos developing to morulae (Matsui et al., 1995), indicating positive effects of IGF-I on embryo development.

The source of IGF-I in the oviduct is either as a serum transudate or de novo synthesis in the oviductal epithelium. Wiseman et al. (1992) suggest that the majority of IGF-I in the oviduct fluid is from oviduct epithelial cell synthesis because they found no correlation between serum levels of IGF-I and oviduct fluid levels of IGF-I throughout the estrous cycle in the pig. These authors also showed that oviduct cells in culture produce IGF-I in the pig. IGF-I release by oviductal cells in vitro has been established in the bovine (Xia et al., 1996a; Winger et al., 1997), but was undetectable in human oviductal cell culture media (Lai et al., 1996). Lee et al. (1992) also found very low secretion of IGF-I by cultured oviductal epithelial cells at d 2 of pregnancy, concluding that the role of IGF-I is minor. However, this conclusion presumes that the oviductal cells are the only source of IGF-I. Serum is another source in the oviduct fluid and may be a contributor of IGF-I in oviduct fluid. IGF-I is present in oviductal fluid of the pig, and its concentration is highest at estrus (Wiseman et al., 1992; Buhi et al., 1997), implicating active secretion of IGF-I at this time due to the influence of estrogen. Cultured oviductal cells have been shown to express IGF-I in the human (Pfeifer and Chegini, 1994), pig (Lee et al., 1992), and sheep (Watson et al., 1994). Expression of IGF-I is coincident with the late follicular phase in the sheep (Stevenson and Wathes, 1996) and is highest before ovulation in the marmoset monkey (Gabler et al., 1998), suggesting its expression is induced by estrogen. However, in the cow, mRNA expression of IGF-I was highest after ovulation (Schmidt et al.,1994), suggesting species differences in IGF-I expression, or differences in hormonal regulation.

Expression of IGF-I in the oviduct is believed to be under hormonal control, as estrogen induces IGF-I expression in the porcine uterus (Simmen et al., 1990). Progesterone had similar effects, although the authors suggest that estrogen and progesterone might be acting through different mechanisms. Increases in endometrial IGF-I expression and IGF-I peptide in uterine fluid are concomitant with maximal estrogen production by embryos at d 12 of pregnancy (Letcher et al., 1989, Green et al., 1995). Responsiveness of endometrial IGF-I to blastocyst estrogen may serve to modulate embryonic $P450_{arom}$ mRNA abundance (Green et al., 1995), so that IGF-I and IGF-II can cause proliferation and differentiation of the endometrium in preparation for blastocyst implantation (Simmen et al., 1992). However, Dalton and co-workers (1994) found that ovariectomy did not change the levels of IGF-I mRNA in the oviduct of the mouse, and Geisert et al. (1991a) found no cyclic changes in bovine endometrial IGF-I expression, arguing against a major role of steroids in the regulation of IGF-I expression. IGF-I expression increases in the liver in response to GH treatment in the pig, but not in the uterus or oviduct (Dunaiski et al., 1999), indicating that IGF-I expression in the reproductive tract is differentially regulated from the liver. In addition, GH treatment of cows for 16 days did not increase oviduct and uterine mRNA expression for IGF-I, IGF binding protein (IGFBP) -2 and IGFBP –3 (Kirby et al., 1996). In contrast, addition of GH to culture medium increased IGF-I production by bovine oviductal cells (Makarevich and Sirotkin, 1997), demonstrating differential effects of GH in vitro and in vivo.

Although the presence of IGF-I in oviduct fluid suggests a role in the early cleaving embryo, initiation of expression of IGF-I receptors differs across species. In the mouse, these receptors are not present until the morula stage (Schultz and Heyner, 1993); however, in the bovine (Winger et al., 1997) and sheep (Watson et al., 1994) the receptors are expressed very early at the 1-cell stage. Interestingly, in the mouse, where expression of IGF-I receptor is later in development, IGF-I has inhibitory effects on the mouse embryo at the 8-cell stage and then positive effects at the blastocyst stage (Shi et al., 1994). Using immunohistochemical techniques, IGF-I receptors were not detected on embryos between d 4 to 10 of pregnancy in the pig (Chastant et al., 1994), but receptor-mediated binding of IGF-I to the trophectoderm of porcine embryos was observed at d 15 to 19 of pregnancy (Corps et al., 1990).

However, Green et al. (1995) found that the IGF-I receptor was constituitively expressed by conceptuses from d 8 to 15 of pregnancy, but they did not examine cleavage stage embryos. Although this evidence suggests that IGF-I does not affect the early cleavage stage embryo in the pig, it is present at highest concentrations in oviduct fluid at estrus (Wiseman et al., 1992). Therefore, through paracrine or autocrine actions on epithelial cells, IGF-I also may act in proliferation and differentiation of oviduct epithelium at a time when gametes or embryos are present.

In situ hybridization on the human oviduct shows that the IGF-I receptor is present on tubal epithelial cells, coincident with IGF-I expression in the human (Pfeifer and Chegini, 1994), demonstrating potential autocrine or paracrine interactions. Also, the IGF-I receptor is expressed in sheep oviductal cell culture (Watson et al., 1994), and was highest in the early luteal phase in the sheep oviductal mucosa and muscularis (Stevenson and Wathes, 1996).

The last growth factor in this family is IGF-II, and it reacts with another receptor, the type II/ Mannose-6-phosphate receptor (see review by Kaye, 1997). However, IGF-II also reacts with the same affinity to the IGF-I receptor and this is how it is thought to exert its growth factor actions. IGF-I1 is present in oviduct fluid in the pig (Wiseman et al., 1992), and its concentrations are highest at estrus. IGF-II is a more potent mitogen than IGF-I in blastocyst-stage mouse embryos, as it stimulates the synthesis of more proteins than IGF-I when added to culture medium (Shi et al., 1994). IGF-II mRNA is expressed in bovine (Xia et al., 1996; Winger et al., 1997) and ovine (Watson et al., 1994) oviductal cell cultures and also released from porcine oviductal explants (Wiseman et al., 1992). In these studies, IGF-II release by oviduct cells was higher than release of IGF-I, indicating that either embryos or the oviductal epithelium may require IGF-II at this time. Unlike IGF-I, IGF-II in the porcine uterus was not hormonally responsive (Simmen et al., 1990), suggesting that the synthesis and release of IGF-II may not be affected by alterations in ovarian hormones. However, Stevenson and Wathes (1996) showed that expression of IGF-II exhibited no temporal changes in the sheep oviduct in the muscularis, yet was higher during the late-follicular and early-luteal stages in the mucosa. Interestingly, Stevenson and co-workers (1994) did not detect IGF-II in the sheep uterus although it was concurrently detected in the oviduct, which points to locally produced oviductal factors being responsible for its regulation. It is probable that IGF-II has multiple roles in the oviduct, affecting oviductal cells (Simmen et al., 1992) and possibly the developing embryo. The IGF-II receptor has been detected on the mouse embryo at the 2-cell stage (Rappolee et al., 1990) and on days 8 and 10 porcine embryos (Chastant et al., 1994). Antisense for IGF-II reduced the rate of development of mouse embryos to the blastocyst stage (Rappolee et al., 1992). The embryo can also produce IGF-II, and embryonic IGF-II stimulates protein synthesis, blastocyst formation and cell proliferation as demonstrated in a series of studies by Kaye and co-workers (in review by Kaye, 1997).

The biological action of IGFs are modulated through the high affinity receptors already discussed, but also through a unique group of proteins that regulate IGF-I availability, the IGF binding proteins (IGFBP). From a review by Jones and Clemmons (1995), the IGFBPs regulate IGF biological activity through 1) transportation of IGFs, 2) extending IGF half life, 3) contribution to tissue and cell-specific distribution of IGFs, and 4) modulation and potentiation of IGF-I action with receptors. Little is known about the specific functions of these binding proteins in the oviduct, but in the ovary IGFBP–2, -4, and –5 are associated with follicular atresia (Grimes et al., 1994; de la Sota et al., 1996). IGFBP-4 is the only binding protein to only have inhibitory actions under all experimental conditions (see review by Jones and Clemmons, 1995), and may possibly serve to protect cells from overstimulation by IGFs or to allow activation of alternate pathways for IGF action.

In situ hybridization studies have shown highest staining of immunoreactive IGFBP-1, followed by IGFBP-4, IGFBP-2, and IGFBP-3 in the human Fallopian tube (Pfeifer and Chegini, 1994). Highest staining was associated with the later proliferative and early secretory stages of the menstrual cycle. The authors concluded that in the human, the coordinate expression of IGFBP-3 and

IGF-I functions to retain IGF in tissue. Similarly, Stevenson and Wathes (1996) from their results in the sheep, suggested that the coordinate expression of IGF-I, IGF-II, IGF-I receptor, and IGFBP-3, demonstrated a possible role in providing an oviductal environment for conception and early embryonic growth and metabolism. Lai et al. (1996) compared the levels of binding proteins in human oviductal cell cultures with or without mouse embryos and found that embryo culture alone produced no IGFBP-3, yet oviduct plus embryo culture produced higher levels of IGFBP-3 than oviductal culture alone. This suggested that the oviductal cells are the source of IGFBP-3 and there is an interaction with the embryo. They also showed that increased levels of IGFBP-3 in human oviductal cell co-culture improved the development of mouse embryos, providing a positive role for this binding protein in embryonic development. IGFBP-3 is the major binding protein found in serum and elevated levels are associated with follicular growth in the ovary (Grimes et al., 1994), suggesting a positive role for this binding protein in other systems as well.

In the cow, using RT-PCR techniques, IGFBP-1 and IGFBP-6 were not detected and the highest signal was for IGFBP-3 and IGFBP-4, followed by IGFBP-5 and IGFBP-2 (Winger et al., 1997). Stevenson and Wathes (1996) examined temporal changes in sheep oviduct IGFBP using in situ hybridization and found that IGFBP-3 was highest at late follicular stages and IGFBP-4 was highest at early and mid-luteal stages. Interestingly, there are species differences in these binding proteins, as IGFBP-1 was detected in the human Fallopian tube, but not the cow oviduct, and IGFBP-2 was not detected in the sheep oviduct, yet was present in bovine and human oviducts. In the pig, IGFBP 1-4 have been immunolocalized on oviductal epithelial cells from d 0 to 12 of pregnancy, with higher staining for IGFBP 1-2 than IGFBP 3-4 (Buhi et al., 1997). IGFBP-1, IGFBP–2 and IGFBP-3 levels in culture media of human endometrial stromal cells are significantly increased following progesterone administration (Bell et al., 1991), demonstrating steroid responsiveness of IGFBPs along with IGFs.

These binding proteins are also expressed by the early embryo (Winger et al., 1997) in the cow, demonstrating a potential for the embryo to modulate IGFs for its own use. The IGF circuit has not yet been fully characterized between the embryo and the oviduct. However, the presence of the IGFs, their receptors and IGFBPs have been established in the oviduct epithelium, in the embryo and in oviduct fluid, indicating a possible involvement of this family of growth factors in embryonic development.

Adding to this complex system, IGFBP 2-5 have specific serine proteases (as extensively reviewed by Jones and Clemmons, 1995), which have differential effects on the action of these binding proteins after proteolysis. When IGFBP-4 is subjected to proteolysis by its serine protease, it is unable to bind to IGF-I, removing the inhibitory effect of IGFBP-4 on IGF-I action. In addition, it has been shown that IGFBP-3 can bind to the cell surface and inhibit cell growth independent of IGF-I (McCusker et al., 1991). Recently, Lee et al. (1998) showed that elongating pig conceptuses induce IGFBP-3 proteolysis through a serine protease, which may increase the intrauterine bioavailability of IGFs. The presence of these serine proteases has not been characterized in the oviduct, increasing the complexity of the IGF system, and demonstrating how much more research is needed to understand this system.

E.4.2.2 *Epidermal growth factor (EGF) and transforming growth factor (TGF)-α family*

The receptor for epidermal growth factor (EGF) and its ligand EGF, transforming growth factor alpha (TGF-α) and amphiregulin (Ar) are expressed in porcine oviduct (Kennedy et al., 1994; Swanchara et al., 1995; Buhi et al., 1997; Wollenhaupt et al., 1997) with the exception of heparin-binding epidermal-like growth factor (HB-EGF). They are localized to the epithelial cells (Kennedy et al., 1994; Wollenhaupt et al., 1997) and EGF receptor concentrations are highest at d 1 of the estrous cycle in the porcine oviduct, suggestive of a role of EGF or TGF-α in the regulation of oviductal cellular events (Wollenhaupt et al., 1997). In the mouse, mRNA was found in the oviduct secretory epithelial cells for TGFα, HB-EGF, and EGF (Dalton et al., 1994). Also, the human oviduct epithelium contained transcripts and protein for EGF and TGF-α during the late follicular and luteal phases of the cycle (Morishige et al., 1993; Chegini, 1996). Interestingly, growth factor transcripts localized in secretory epithelial cells decreased after ovariectomy, whereas growth factors localized within the muscle did not change, indicating a selective role of steroids in the secretory activity of the oviduct. The EGF and TGF-α proteins are present in porcine oviduct flushings (Buhi et al., 1997), but EGF concentration is not different between d 0 and 12 of pregnancy. Swanchara et al. (1995) also detected EGF in porcine oviduct flushings and the EGF receptor in oviduct tissue at d 2 of the estrous cycle. Buhi et al. (1997) showed that TGF-α in oviduct fluid was highest at d 0 of the cycle, and increased in response to estrogen treatment in ovariectomized gilts. Both EGF and TGF-α expression increased in relation to serum estradiol not progesterone in the human oviduct (Morishige et al., 1993), providing additional evidence of hormonal control.

Rappolee et al. (1990), Schultz and Heyner (1993), Chegini (1996), and Kaye (1997) have reviewed data on the EGF family and their roles in preimplantation embryonic development. Both EGF and TGF-α interact with the EGF receptor (Massague, 1983), and the EGF receptor encoded by the embryonic genome has been detected on mouse embryos at the 4-8 cell stage. However, maternal mRNA for the EGF receptor has been found in the zygote and at the 2-cell stage, prior to embryonic genome activation using RT-PCR (Wiley et al., 1992). The presence of the receptor on embryos implies a role of EGF or TGF-α in the developing embryo, however, TGF-α transcripts have been detected on the ovine embryo throughout preimplantation development (Watson et al., 1994), suggesting that the embryo is not dependent on external sources of TGF-α for development. On the other hand, these authors did not find transcripts for EGF at any time on the ovine embryo, implying that EGF is required from external sources, such as the oviduct. TGF-α may be acting in autocrine regulation, being produced by the embryo and acting on embryonic receptors, and the full effect on embryonic development may require EGF and/or TGF-α from the maternal reproductive tract (Paria and Dey, 1990). This conclusion is in agreement with Kaye (1997), who suggests that TGF-α is derived from the inner cell mass and acts on EGF receptors in a paracrine circuit to stimulate trophectoderm functions. In support of this hypothesis, mouse embryos with the EGF gene deleted are observed to have regressed inner cell masses and fail to implant (Sibilia and Wagner, 1995).

When added to culture media, EGF accelerated the rate of 2-cell mouse embryos reaching the blastocyst stage and subsequent hatching from the zona (Paria and Dey, 1990), providing evidence for embryotrophic effects of this growth factor. In addition, EGF promotes blastocyst formation and stimulation of mouse embryo development in cooperation with TGF-α, as incubation with either anti-TGF-α or anti-EGF abolishes the beneficial effect of co-culture with human oviductal cells (Morishige et al., 1993). In the pig, the effects of EGF or TGF-α have not been examined in the early cleavage stage embryo; however, Corps et al. (1990) found functional receptors for EGF on trophectoderm cells in the d 15 to 19 pig embryo.

E.4.2.3 *Transforming growth factor (TGF)-β and Fibroblast growth factor (FGF) families*

TGF-ß2 transcripts have been detected in the bovine oviductal epithelium (Watson et al., 1992), TGF-ß1 in ovine oviduct cell culture medium (Watson et al., 1994) and TGF-ß1, TGF-ß2, TGF-ß3 and TGF-ß type II receptor were detected using immunocytochemistry techniques in the porcine oviduct (Buhi et al., 1997). The staining intensity for TGF-ß1 and the TGF-ß type II receptor was increased during pro-estrus, providing evidence for hormonal control of their expression (Buhi et al., 1997). In contrast, TGF-ß mRNA levels did not decrease in response to ovariectomy in the mouse oviduct, indicating lack of an ovarian steroid influence on TGF-ß regulation (Dalton et al., 1994). TGF-ß 1-3 are expressed in the human (Zhao et al., 1994) and mouse (Dalton et al., 1994) oviducts, and TGF-ß type I-III receptor expression and protein was also detected in human Fallopian tube (Zhao et al., 1994). In d 10 to 14 porcine conceptuses, mRNA for TGF-ß 1-3 was detected in many embryonic membranes, and expression increased from d 12 to 14 of gestation (Gupta et al., 1998). Although TGF-ß expression was not examined in early cleavage stages, this data is suggestive of roles for TGF-ßs during the peri-implantation period. However, since the embryo is capable of expressing TGF-ß, expression of these growth factors in the reproductive tract may play autocrine or paracrine functions to regulate the oviductal and uterine epithelium.

Basic FGF mRNA was detected in ovine oviductal cell culture media and in the ovine embryo, and mRNA for basic FGF decreased from the 1-cell to the blastocyst stage, suggesting a maternal source of the transcript (Watson et al., 1994).

Evidence exists for embryotrophic effects of TGF-ß, as addition of TGF-ß with basic FGF promoted formation of bovine blastocysts in vitro past the 8-cell block (Larson et al., 1992a). It is possible that TGF-ß promotes the synthesis of extracellular matrix factors, while basic FGF acts as a mitogen (as reviewed by Schultz and Heyner, 1993). However, Chegini (1996) in a review of the available literature, indicated that these growth factors do not correlate well between large animals, rodents and humans, and, therefore, we must keep in mind species differences when elucidating growth factor circuits in the oviduct and their potential role. Currently, more research need to be done with regard to expression of receptors in early embryos, and concurrent expression in the oviduct of these factors to establish a specific role of these factors in embryonic development.

E.4.2.4 *Other growth factors*

Platelet-derived growth factor (PDGF) is a major mitogen in serum, and consists of two subunits α and ß, which after processing, form three major isoforms (as reviewed by Schultz and Heyner, 1993 and Chegini, 1996). PDGF-α and –ß receptor genes are expressed throughout bovine preimplantation embryonic development (Watson et al., 1992), but thus far no studies have shown expression of PDGF in the pig embryo or oviduct. PDGF, when added to 2-cell bovine embryos increased the percent of embryos developing beyond the 16-cell stage (Larson et al., 1992b), demonstrating potential embryotrophic effects of this growth factor. Interestingly, in the same study, if PDGF was added at the 16-cell stage, it reduced the percentage of bovine embryos reaching blastocyst stage, suggesting that PDGF has differential effects at specific stages. Similarly, incubation of bovine embryos with PDGF increased the proportion of embryos developing to the 8-cell and morula stage (Thibodeaux et al., 1993). Also, addition of anti-PDGF, or incubation with bovine oviductal epithelial cells and anti-PDGF, reduced the proportion of embryos reaching these stages compared to PDGF incubation, and also reduced the inner cell mass. Therefore, PDGF has embryotrophic effects on the bovine embryo, and the addition of PDGF antibodies appears to reduce embryo development by inhibiting PDGF action.

Colony- stimulating factor (CSF) –1 mRNA has been detected in pig oviduct (Tuo et al., 1995), and in the mouse both CSF-1 mRNA and protein increase to 100-fold higher levels in d 14 pregnant uterine epithelium compared to tissue from non-pregnant mice, then decrease thereafter (Arceci et al., 1989). CSF-1 first becomes detectable at d 3 (Arceci et al., 1989), just before the mouse embryo implants, and is implicated with a role in implantation in the pig (see review in Jiang, 1999). CSF-1 appears to be regulated by estrogen and progesterone (Pollard et al., 1987), and thus may play a role in embryonic survival at the implantation stage in the pig. The extent to which the oviduct synthesis of CSF-1 interacts with the developing embryo during its residence in the oviduct remains to be determined.

A secreted glycoprotein, leukemia inhibitory factor (LIF) has high expression in the human distal ampulla and is speculated to have a role in early embryonic development (Keltz et al., 1996). In a review by Schultz and Heyner (1993), they suggest that uterine LIF may be involved in the regulation of cellular proliferation of the developing embryo, as LIF is reported to inhibit embryonic stem cell differentiation. LIF expression is greatly increased in response to oviductal cytokines and growth factors such as TGF-β1 and EGF in the stroma, suggesting a role in tubal implantation (Keltz et al., 1996) and possibly in assisting embryonic implantation at later stages. The synthesis of LIF from cultured bovine oviductal epithelial cells is increased in the presence of 17ß-estradiol and TNF-α, however, this effect is not receptor mediated (Reinhart et al., 1998).

Macrophage migration inhibitory factor (MIF) mRNA was also found to be expressed in the mouse oviduct, yet is found in the tunica muscularis, not the secretory cells, and it does not fluctuate with the estrous cycle (Suzuki, 1996). The MIF factor is probably responsible for activation of macrophages in the oviduct tissue and not directly related to embryonic development.

E.4.2.5. *Cytokines*

The cytokines, interleukin-1 (IL-1) α and β, neutrophils, IL-1β macrophages or eosinophils were not found to be expressed in the oviduct of the mouse, but the oviduct does not undergo an inflammatory response to mating (Dalton et al., 1994). In the human, IL-10 was in high concentrations in the Fallopian tube suggesting its role as an immunosuppressant (Srivastava et al., 1996). Hook and Hafez (1968) found lymphoblast-like cells which migrate through to the lumen and appear to undergo mitosis during the estrus period in eight of eight species examined, suggesting immune function is important for certain regulatory events in the oviduct.

E.4.2.6 *Proteins*

Other families of oviduct derived proteins that are of interest are the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Although this family has been implicated in implantation (Menino et al., 1997; Jiang, 1999), they also exist in the oviduct environment and may have a potential role in the oviduct and the developing embryo.

Satoh et al. (1994) found that bovine oviductal epithelial cells secreted a tissue inhibitor of metalloproteinase-1 (TIMP-1) which in vitro stimulated embryogenesis. TIMP-1 is synthesized and secreted de novo by all segments of the porcine oviduct, however, it is highest at d 2 of the estrous cycle in the isthmus (Buhi et al., 1997b). This study also showed that TIMP-1 protein was present throughout the estrous cycle in oviduct flushings, and although highest concentrations were observed at estrus. There were no differences after ovariectomy or steroid-treatment, yet the Meishan breed had lower concentrations than Large White breed. They also showed that TIMP-1 mRNA was present in all sections of the oviduct, and also underwent cyclic changes, and major changes in TIMP-1 can be attributed to the isthmus between d 0 to 2 of the cycle. Lastly, Buhi et al. (1997b) also localized TIMP-1 on porcine luminal oviductal epithelium in all segments, and highest staining was seen at d 0 of the estrous cycle. Satoh et al. (1994) suggested that TIMP-1 functions as an embryogenic simulator on in vitro fertilized bovine embryos.

Plasminogen activator inhibitor (PAI)-1 is also present in the early pregnant pig oviduct (Kouba et al., 1997) and this inhibitor as well as the TIMPs, may function to regulate the oviductal microenvironment and to protect oocytes and embryos from proteolytic damage and enzymes in the lumen (Buhi et al., 1997).

Protection of the uterine lining has been indicated for PA inhibitor secretion by the uterus at around implantation in the pig (Fazleabas et al., 1983), suggesting a similar role in protection of the oviduct epithelium from enzymatic damage.

F CONCLUSION

This chapter has provided convincing evidence that normal gamete transport and maturation, fertilization, and early embryonic development are dependent on oviduct function. Also, we know that estrogen, progesterone, and their relative levels influence virtually every aspect of oviduct function. The oviduct is especially sensitive to these steroid concentrations due to the sub-ovarian counter-current exchange system, as coordinate function of the oviduct is intimately associated with the ovarian steroid concentrations prevailing in the local oviductal circulation. The oviduct is also an active secretory organ, synthesizing and secreting specialized proteins and growth factors that associate with the gametes and developing embryos. These oviductal proteins and growth factors are also under the influence of ovarian steroids, and therefore, we can appreciate how important the oviductal environment is to embryonic viability and development. Therefore, returning to a consideration of embryonic loss as a determining factor in litter size in the pig, embryonic diversity and the appropriate steroid priming of the uterus are documented as two factors that contribute to embryonic loss. However, the literature reviewed above suggests that the oviduct also plays a major role in fertilization failure and embryonic viability, and therefore contributes to embryonic loss. The following chapters detail investigations into the possible role of the oviduct in embryonic survival in the gilt.

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CHAPTER 2

TEMPORAL RELATIONSHIPS IN PERIPHERAL AND OVIDUCTAL PLASMA PROGESTERONE CONCENTRATIONS IN THE IMMEDIATE POST-OVULATORY PERIOD IN GILTS[1]

INTRODUCTION

Current pig production trends place a great demand on reproductive capability in gilts, as they constitute a substantial proportion of the breeding herd. While still growing at a rapid rate they are required to produce large litter sizes. The challenge is to create nutritional regimens that optimize litter size and reproductive performance. An important component of litter size is embryonic survival rate, as embryonic loss in gilts and primiparous sows up to d 30 of pregnancy is around 30 % (Foxcroft, 1997). The practice of 'flush feeding' (feeding ad libitum before ovulation to maximize ovulation rate) can be detrimental to embryonic survival if continued into early pregnancy (reviewed by den Hartog and van Kempen, 1980; Foxcroft, 1997). Dyck and Strain (1983) reported that high feed intakes from d 1 to 10 of pregnancy, but not d 10 to 20, were responsible for reduced embryonic survival at d 30 in gilts. Although controversial, others have also reported that a high plane of feeding in early pregnancy is detrimental to embryonic survival in pigs (Ashworth et al., 1991; Pharazyn, 1992) and reduces pregnancy rate in sheep (Parr et al., 1982; Parr et al., 1987). In a further study by Jindal et al. (1996), gilts that were reduced to NRC requirements for energy maintenance (M; 1.5 X M) immediately after breeding on d 1 of gestation had higher embryonic survival at d 28 than their counterparts fed at 2.0 X M, (84.5% vs. 64.5%). In that same study, a third group of gilts reduced to 1.5 X M at d 3 of pregnancy, exhibited an intermediate level of embryonic survival, consistent with the lack of an effect of nutritional treatments imposed at d 3 of pregnancy on embryonic survival reported by Pharazyn et al. (1991a). A critical window, therefore, appears to exist during which high intake can alter embryonic survival.

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[1] The results of this study were presented in the review of Foxcroft GR, Dixon WT, Jiang L, Novak S, Mao J, Almeida FRCL 2000. Insights into conceptus-reproductive tract interactions. American Society of Animal Science, Biennial Reviews of Reproduction, in press.

Reduced embryonic survival has been associated with lower plasma progesterone concentrations in the first few days of pregnancy and a delay in the timing of the rise in plasma progesterone concentrations in the pig (Pharazyn, 1992; Jindal et al., 1996; Jindal et al., 1997) and in the ewe (Ashworth et al., 1989). Embryonic survival can in part be restored with exogenous progesterone injections in gilts maintained on a high plane of nutrition after mating (Ashworth et al., 1991; Jindal et al., 1997), and improves pregnancy rate in the ewe in similar circumstances (Parr et al., 1987), implicating progesterone as a mediator of nutritional effects on embryonic survival. The initial rise in progesterone is also earlier in Meishan compared to Large White gilts (Hunter et al., 1996) and is thought to be a factor in the fertility of the Meishan pig. Although the mechanism by which a high feeding level affects circulating progesterone concentrations is not clear, it may act by increasing metabolic clearance rate of progesterone by the liver (Prime and Symonds, 1993). Alternatively, reduced progesterone production from the ovary could be a factor. In turn, reduced peripheral plasma progesterone concentrations in early pregnancy may affect the steroid priming of the uterus and the secretion of progesterone–induced proteins implicated in embryonic viability and implantation (as reviewed by Roberts and Bazer, 1988; Roberts et al., 1993). Also, the synchrony of the embryos and uterus may be affected, resulting in embryonic loss (Pope, 1988).

In contrast to the uterus, priming of the oviduct is subject to much higher steroid concentrations on account of the counter-current multiplier system located immediately below the ovary (Krzymowski et al, 1982a; Hunter et al., 1983). Counter-current transfer of steroids from the ovarian vein to ovarian and oviductal arteries increases progesterone concentrations 10-fold compared to peripheral concentrations (Pharazyn et al., 1991b). Therefore, if ovarian production of progesterone were reduced through nutritional feeding regimens, this would adversely affect the unique hormone milieu of the oviduct. Oviduct contractility (Nozaki and Ito, 1987), oviduct fluid formation (Leese, 1988), and synthesis and secretion of oviduct proteins are under hormonal control (Murray, 1992; Buhi et al., 1992). Estrogen- and progesterone-induced oviductal proteins have been characterized in many species, and possibly affect gametes and embryos (see reviews by Nancarrow and Hill, 1995; Buhi et al., 1997; Chapter 1, this thesis). This raises the possibility that alterations in secretion of these proteins and the function of the oviduct may be another potential mechanism by which nutrition affects embryonic survival.

The objective of this study was to use the gilt model established by Jindal et al. (1996) to determine if the lower progesterone concentrations in gilts fed a high feed intake after mating (2.0 X M) were a result of decreased ovarian production of progesterone or an increase in metabolic clearance of this steroid. This was achieved by performing surgery to obtain plasma progesterone concentrations in peripheral, uterine, and oviductal veins, and in the sub-ovarian plexus, at a time when Jindal et al. (1996) and Jindal et al. (1997) reported that peripheral progesterone concentrations were different across treatment groups. If progesterone concentrations were different in the ovarian drainage, as well as being different in the peripheral circulation between normal (N) and high (H) fed groups, this would imply that nutritional treatment affected progesterone production by the corpora lutea. However, if progesterone concentrations were similar in the ovarian drainage, but different in peripheral plasma across treatment groups, this would imply that metabolic clearance rate of progesterone is affected. The physiological significance of this experimental design is that it provides insight into the mechanisms of progesterone-mediated effects on embryonic survival. If ovarian production of progesterone were affected by treatment, then the steroid priming of the oviductal environment becomes a factor in embryonic survival due to the counter-current multiplier system. However, if only peripheral concentrations of progesterone are affected, then the steroid-priming of the uterus becomes more important, due to the fact that the uterus is only exposed to peripheral steroid concentrations in the pig (Pharazyn et al., 1991b).

MATERIALS AND METHODS

Animals

Twenty first or second cycle Camborough x Canabrid terminal line gilts (Pig Improvement (Canada) Ltd.) were used in this experiment and housed at the University of Alberta Swine Research Unit, in barns with a totally controlled environment. All animals were cared for in accordance with CCAC Guidelines and with authorization from the Faculty Animal Policy and Welfare Committee. Gilts were individually fed twice daily a total of 2.0 X Energy Maintenance (M) requirements of a standard barley-wheat-soybean meal grower diet containing 14.16 MJ DE/kg and 14% CP. Feed allowances were calculated based on metabolic body weight (BW $kg^{.75}$) within 10 kg body weight ranges, to provide a maintenance energy allowance of 461 kJ DE/kg of metabolic BW. Gilts had ad libitum access to water. Estrus detection was carried out every 12 h starting at d 18 of the cycle using backpressure testing during fenceline contact with a vasectomized boar. The onset of estrus (d 0 of pregnancy) was determined as the time of occurrence of a standing reflex in the presence of a boar, minus 6 h. To minimize any effect of boar on embryonic development, gilts were artificially inseminated with fresh, pooled semen from the same three boars 12 and 24 h after detection of estrus. All inseminations were carried out by the same, trained person. Immediately after the last insemination (d 1 of pregnancy), gilts were matched for weight, and randomly assigned within a weight-pair to one of two feeding treatments. Feed intake was either reduced to 1.5 X M (N group) based on NRC (1988) recommendations for gestation requirements, or continued at 2.0 X M (H group) until d 10 of pregnancy. At d 11, intake in H gilts was also reduced to 1.5 X M.

Blood Sampling

At d 16 of the estrous cycle, gilts were surgically fitted with indwelling jugular catheters via the superficial cephalic vein under general anesthesia (Cosgrove et al., 1993). From d 18 until d 5 of pregnancy, or until the time of a second surgery, 5 ml blood samples were taken every 4 h to determine timing of the preovulatory peak in plasma LH, and plasma progesterone concentrations. At 72 h after onset of estrus, heparinized blood samples from the jugular, uterine and the oviduct veins, and mixed arterial and venous blood from the ovarian pedicle were taken under general anesthesia. All blood samples were centrifuged (2200 X g, 4°C) and plasma separated and stored at –30°C until assayed for progesterone and LH concentrations.

Embryo and Uterine tissue collection

Pair-matched gilts were slaughtered between d 9 to 17 of pregnancy at a local abattoir. Immediately after slaughter, reproductive tracts were removed from each gilt. Corpora lutea were counted on both ovaries as a measure of ovulation rate. As part of research collaboration with other colleagues, the oviduct and uterus were dissected free of mesosalpinx and mesenteric tissue and the uterus was clamped tightly at 1 cm from the utero-tubal junction. Each uterine horn was also clamped tightly near the cervix and dissected free from the rest of the reproductive tract. Each uterus was then hung from the uterotubal junction, and flushed with 10 ml of physiological saline. The flushing technique included insertion of a blunted 18g needle attached to a syringe into the uterine lumen just below the uterotubal junction. The fluid was gently massaged down towards the cervical end of the uterus and collected into a sterile Petri dish. Any embryos in the first 10 ml flush were recovered and the 10 ml of uterine flushings were poured into a sterile 15 ml Falcon tube and snap frozen in liquid nitrogen. The uterus was then flushed twice with 60 ml saline to collect remaining embryos into a clean beaker. The embryos were again recovered from flushings and the flushings discarded. All embryos from the first uterine horn to be flushed were designated for tissue fixation and were put into 15 ml Falcon tubes containing Buffered Paraformaldehyde (Harvey et al., 1995). A sample of uterine tissue from that uterine horn was taken using blunt dissection and also placed in fixative. The second uterine horn to be flushed was designated for mRNA analysis, and embryos, 10 ml uterine flushings, and uterine tissue were immediately snap frozen in liquid nitrogen.

Embryo and uterine tissue analysis

Embryonic and uterine tissue designated for fixation were processed as per Harvey et al. (1995), and were shipped in 70% EtOH to the laboratory of Dr. Gil Schultz, University of Calgary for further analysis[2]. Embryos and uterine tissue designated for mRNA analysis were stored at –70°C until analysis, as part of a collaborative study with L. Jiang in our laboratory[3].

Radioimmunoassays

Plasma LH concentrations were determined in duplicate using the homologous double antibody radioimmunoassay previously described by Cosgrove et al. (1991). 200µl of plasma were assayed and the intra- and inter-assay CV were 11.5 % and 11.9 %, respectively. Assay sensitivity defined as 96 % of Total Binding was 0.02 ng/ml.

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[2] Analysis of samples appears in: Menino AR Jr, Hogan A, Schultz GA, Novak S, Dixon W, Foxcroft GH 1997. Expression of proteinases and proteinase inhibitors during embryo-uterine contact in the pig. Developmental Genetics. 21: 68-74.

[3] Analysis of samples appears in: Jiang L. Gene expression in blastocyst and endometrium. Edmonton, Canada: University of Alberta; 1999. Thesis. Also in: Jiang L, Novak S, Foxcroft GR, Dixon WT. Expression of matrix metalloproteinases and their regulators during porcine peri-implantation development. Placenta, submitted for publication; and Jiang L, Novak S, Ramsoondar J, Foxcroft GR, Dixon WT. Macrophage colony-stimulating factor (CSF-1)/c-fms is an important autocrine and paracrine growth factor-receptor pathway for regulating proliferation of porcine trophoblast cells. Molecular Reproduction and Development, submitted for publication.

Plasma progesterone concentrations were determined using an established radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corporation, U.S.A.), previously validated for use with porcine plasma without extraction (Mao and Foxcroft, 1998). Oviductal plasma samples were pre-diluted 10- and 50-fold, and ovarian pedicle samples were diluted 100- to 500- fold with zero calibrator provided with the kit before being assayed in duplicate. The sensitivity of the assay, defined as 95.2 % of total binding, was 0.098 ng/ml. The intra- and inter-assay CV were 3.1 % and 9.8 %, respectively.

Statistical Analysis

Normal distribution of data was checked by the Shapiro-Wilk test in SAS (1990), and plasma progesterone was log transformed to achieve normal distribution. Treatment effects on feed intake and number of corpora lutea were analyzed by the GLM procedure of SAS (1990), using Treatment as the independent variable and the variation across animals as the error term. For evaluation of treatment effects on progesterone concentrations from sequential sampling, repeated measures analysis of variance (SAS, 1990) was used. Plasma progesterone concentrations at each sampling site were analyzed using the GLM procedure of SAS (1990), with time after ovulation as a covariant, to eliminate effects of time on progesterone concentrations. The statistical model included Treatment as the independent variable, progesterone concentrations at each site as dependent variables, Time after LH peak as the covariate, and variance across animals as the error term. Because of unequal sample sizes, Least Squares Difference test was used to compare differences between means. This test was only performed if the statistical model and treatment were both significant ($P<0.05$). All correlations were analyzed using linear regression analysis (SAS 1990). The data are presented as LSM (± SE of LSM).

RESULTS

The average starting weights for gilts before treatment were 130.2 ± 4.36 kg (H) and 137.4 ± 4.36 kg (N). Average feed intakes on treatment was 2.54 ± 0.19 kg (range 1.8 – 2.2 kg) and 2.02 ± 0.17 kg (range 2.2 - 2.8 kg) daily for H and N, respectively.

There was no difference ($P>0.10$) in the interval between onset of estrus and LH peak between treatment groups (7.6 ± 4.4 h and 10.7 ± 4.7 h for H and N, respectively). However, the H group had a lower ovulation rate (13.6 ± 0.72), compared to the N (16.0 ± 0.72) group ($P<0.03$).

Plasma progesterone profiles

The plasma progesterone profiles with respect to LH peak are presented in Figure 2.1. There was no treatment effect on mean progesterone concentrations at any sampling time, or on the timing and rate of rise of progesterone.

Ovarian, oviduct and jugular samples at surgery

Surgeries were performed at 71.0 ± 4.6 h for H gilts and 71.8 ± 4.9 h for N gilts after the LH peak, and there was no difference in this interval between treatments. There was no treatment effect, or time by treatment interaction, on progesterone concentrations obtained from ovarian, oviduct and jugular venous plasma at surgery (Table 2.1). However, there was a significant effect of time ($r=0.79$, $P=0.0001$) on plasma progesterone concentrations in the peripheral circulation (Figure 2.2). Interestingly, there was no effect of time on ovarian and oviductal venous plasma and progesterone concentrations were already high at 48 h after the LH surge and remained fairly constant during the 50-h period. There was high variation among gilts in both oviductal and ovarian progesterone concentrations.

DISCUSSION

In this experiment, there were no differences in plasma progesterone profiles 0 to 96 h after the LH peak, or a difference in timing or rate of rise of progesterone concentrations between the H and N groups. These results, therefore, are not consistent with the results of Jindal et al. (1996) and Jindal et al. (1997) from our laboratory, who used gilts of the same genotype. However, others have failed to observe progesterone-mediated changes in embryonic survival as a result of nutritional manipulation (Parr et al., 1987; Pharazyn, 1992; Cassar et al., 1994). It is evident that nutritional effects on embryonic survival and their associations with progesterone are not consistent and this phenomenon remains controversial.

We currently do not have an explanation for the inconsistency between this study and those of Jindal et al. (1996) and Jindal et al. (1997). One possibility, however, is that the H gilts in this study were fed to 2 X M throughout the previous cycle, and did not experience a change in their feed level after mating. This differs from the experimental design of Jindal and co-workers, as all their gilts were initially fed 2.5 kg of feed/d, irrespective of maintenance energy requirements, and all gilts experienced a change in feed level after mating. In other studies where high fed gilts failed to experience a change in their feed level, the lack of a significant effect on embryonic survival was evident (Dyck, 1991; Pharazyn, 1992). Albeit, in the sheep, it has been argued that increases in feed intake after mating result in lower progesterone concentrations during early pregnancy (Parr et al., 1987), due to increases in metabolic clearance rate. Also, no relationship was observed between the timing and rate of rise of plasma progesterone, and embryonic survival in sows fed the same nutritional regimen (Soede et al., 1994). Therefore, the absence of an increase in feed intake after mating in these models, and in our current study, may explain the lack of an effect of these nutritional regimens on peripheral progesterone concentrations in early pregnancy. This does not, however, necessarily suggest that lowered feed intake after mating does not have beneficial effects on embryonic survival, as there is good evidence to support this in the pig. However, it is too simplistic to assume that peripheral progesterone is the only mediator or indicator of these effects. Although we did not measure embryonic survival in this study, even in the absence of differences in progesterone concentration, we may effectively have altered embryonic survival.

Although progesterone concentrations were not associated with embryonic survival, the interval between onset of estrus to peak estradiol (Blair et al., 1994) and the interval between peak estradiol and the rise in progesterone (Soede et al., 1994) were associated with differences in embryonic survival. Blair et al. (1994), however, suggested that differences in peri-estrus hormone profiles between gilts with high and low embryonic survival could be related to follicular development. Since plane of nutrition has been shown to affect follicular quality (Zak et al., 1997), it is possible that when gilts are fed the same amount of feed irrespective of their metabolic requirements during the estrous cycle, differences are created in follicular quality, steroidogenesis and subsequent progesterone secretion. In the gilt, the influence of differences in nutritional state during the estrous cycle on subsequent embryonic survival and progesterone concentrations (Almeida et al., 2000a) are possibly a reflection of differences in follicular maturation, as postulated by Foxcroft et al. (1987) and Hunter and Weisak (1990). This may be the mechanism involved in embryonic survival differences in studies where gilts were overfed or underfed throughout the cycle before treatment (Dyck and Strain, 1983; Ashworth et al., 1991; Pharazyn, 1992; Jindal et al., 1996; Jindal et al, 1997). Therefore, it is too simplistic to assume that post-ovulatory nutritional effects on the uterine environment are largely involved in these models, when nutritional state before and after mating are not tightly controlled. This may also explain why we did not see a difference in progesterone concentrations in the current study, because we controlled for nutritional state in gilts by feeding 2 X M before ovulation.

Our results also show a difference in ovulation rate between H and N gilts, but we believe that this is not an effect of treatment. All gilts would have been in the same nutritional state before treatment, and treatment was imposed well after the LH surge and around the time of ovulation. The difference between treatments, therefore, appears to be a chance occurrence. Even though this difference existed; we did not correct progesterone concentrations for ovulation rate, as there was no relationship between these two parameters.

Because we did not observe a difference in peri-ovulatory peripheral progesterone concentrations between H and N gilts, we were not able to determine whether differences in peripheral progesterone concentrations in the first few days of pregnancy were due to changes in metabolic clearance rate, or changes in progesterone secretion by the ovary. We did, however, observe a significant relationship of peripheral progesterone concentrations, and time after LH peak. More importantly, this relationship with time after the LH peak did not exist in oviductal or ovarian progesterone concentrations. Although Pharazyn et al. (1991b) was able to show a temporal change in oviductal progesterone concentrations over a period of 12 d, we were unable to demonstrate this because of the short sampling duration of 48 h in this study. We would suspect that as progesterone production rises in the corpora lutea in early pregnancy, these increases would be reflected in the oviductal circulation before the time of sampling in the current study. From our observations, it is evident that the rise in plasma progesterone concentrations in the oviductal circulation occurred earlier than 48 h after the LH peak. In support of this, Eiler and Nalbandov (1977) using frequent sampling of the utero-ovarian vein, showed that progesterone concentrations rose in the utero-ovarian vein 16 h before ovulation. They also showed that the pre-ovulatory follicle produces progesterone, and follicular fluid steroids are secreted into the ovarian vein in large amounts approximately 12 h before ovulation, coincident with the increase of progesterone in the utero-ovarian vein. Therefore, the quality of a follicle, and its associated steroidogenic ability, can influence the oviductal environment in advance of a detected rise in peripheral progesterone concentrations. Also, our data suggests that to observe nutritionally-induced differences in the oviductal environment, one should examine the oviductal environment around ovulation, or immediately before ovulation, when progesterone is rising in the oviductal circulation.

Progesterone concentrations are already high in the oviductal circulation due to the presence of the sub-ovarian counter current system. The oviductal circulation is directly linked to the ovarian vascular drainage through the sub-ovarian counter-current system (see review by Krzymowski et al., 1990), and this system transfers high concentrations of steroids in the ovarian vein to the ovarian and oviductal arteries. As a result, the concentration of progesterone in the oviduct veins is 10-fold higher than in peripheral blood (Pharazyn et al., 1991b; this study). The temporal increase in peripheral progesterone concentrations could simply be due to dilution of high ovarian venous drainage in the peripheral circulation; however, the transfer of progesterone from the ovarian vein to the oviductal vasculature is known to be more complex. There is a large accumulation of steroids in the lymph tissue surrounding the counter-current exchange system (Kotwica et al., 1981; Krzymowski et al., 1982b), which suggests that the concentration of steroids in the oviduct veins may not always reflect the concentration in the ovarian veins. This may explain the lack of a relationship between ovarian and oviductal progesterone concentrations in this study. Another complicating factor determining the concentration of progesterone in the oviductal veins is the action of steroids on the vascular bed, regulating blood flow and changing the counter-current transfer of steroids (Stefanczyk-Krzymowska, 1997).

In the pig, the counter current system does not extend into the uterine arteries (Pharazyn et al., 1991b; this study), unlike the sheep (Weems et al., 1989), which is an important difference when elucidating a role for the oviduct in embryonic loss. It appears that in the pig, the uterine environment will only be affected by peripheral progesterone concentrations after ovulation, whereas oviductal function will be affected during pre-ovulatory follicular development and in the immediate post-ovulatory period. Therefore, the oviduct is unlikely to be an important mediator of embryonic loss in nutritional models where feed intake is changed after mating, as in this study. If changes in metabolic clearance rate are responsible for differences in peripheral progesterone and associated embryonic loss, the oviduct is not directly affected by changes in splanchnic clearance of progesterone. However, in an extreme scenario, Mburu et al. (1998) reported that increased transit rate of embryos from the oviduct to the uterus was responsible for lowered embryonic development in gilts that were fasted after mating. This suggests that progesterone concentrations may have been artificially increased in the oviductal circulation during fasting, as exogenous progesterone administration has been shown to increase transit rate of embryos through the oviduct (Day and Polge, 1968). Fasting may have adversely affected splanchnic blood flow (Prime et al., 1993; Freetly and Ferrell, 1994), and resulted in differential blood flow through the oviductal circulation, culminating in an accumulation of progesterone (Foxcroft, 1997). Another possible explanation, is that increases of progesterone in the peripheral circulation may alter concentrations of adrenergic receptors (Einspanier et al., 1999) to adversely affect oviductal contractions. However, it is unlikely that adverse changes in embryo transport would occur in nutritional models that impose modest feed changes.

Evidence is accumulating for an active role of the oviduct in gamete transport, fertilization and early embryonic development. In addition, oviduct function is affected by alterations in ovarian steroids, suggesting that steroid priming of the oviduct may affect embryonic survival. Differential concentrations of steroids in the oviductal circulation between oviducts within an animal can cause differences in the pattern of oviduct protein secretion (Binelli et al., 1999), oviduct pH (Nichol et al., 1997), and sperm transport (Sultan and Bedford, 1996). Also, unique proteins are secreted by the oviduct in response to estrogen and progesterone during the peri-estrus period and have been implicated in early embryonic development (as reviewed by Nancarrow and Hill, 1995; Buhi et al, 1997). In vitro co-culture experiments with oviduct fluid or oviductal epithelial cells have shown a beneficial effect of co-culture on sperm maturation (Dubuc and Sirard, 1995), oocyte maturation

(Broermann et al., 1989; Nagai and Moor, 1990; Kim et al., 1996), fertilization (Way et al., 1997) and blastocyst development (Gandolfi and Moor, 1987; Walker et al., 1996; Xia et al., 1996). It is, therefore, possible that the oviduct plays a major role in embryonic survival by affecting gamete transport, fertilization and early embryonic development. Alterations in this sequence of events may also contribute to increased asynchrony between embryos and the uterus which has been implicated in embryonic loss in the pig (see review by Pope, 1988). If steroid priming of the oviduct is affected during follicular development in nutritionally induced embryonic loss, then the oviduct becomes a potential factor in mediating this embryonic loss.

In conclusion, the objective of this study was to further elucidate the mechanism which results in nutritionally-induced differences in peripheral progesterone concentrations during the peri-ovulatory period that have been associated with changes in embryonic survival. However, due to modifications in pre-treatment feeding, we may have inadvertently altered the model and consequently found no differences in progesterone concentrations between treatment groups during the first few days of pregnancy. However, we did find that peripheral, but not oviductal or ovarian progesterone concentrations, were correlated with time for both H and N groups. This suggests that steroid priming of the oviduct environment could affect embryonic survival by a different mechanism than the uterine environment. The evidence presented here still allows the possibility that nutritional effects on circulating progesterone and/or embryonic survival could be mediated by the quality of the oviductal environment. Our future studies will look at changes in the oviductal environment in controlled situations to gain insight into the responsiveness of the oviduct to ovarian steroids, so we can ultimately determine if the oviduct is implicated in nutritionally-induced differences in embryonic survival.

Table 2.1. Least Squares Means (± SEM) of plasma progesterone concentrations (ng/ml) at surgery around 70 hours after LH peak. There were no differences (P<0.05) between N and H treatments.

Treatment	Jugular	Oviduct	Ovarian
H	8.26 ± 0.7	64.6 ± 23.0	1283.1 ± 263.5
N	9.11 ± 0.7	47.2 ± 24.2	861.9 ± 277.7

Figure 2.1. Profiles of peripheral progesterone concentrations standardized to time after the pre-ovulatory LH peak. Least squares means are presented; N = 10 for each group.

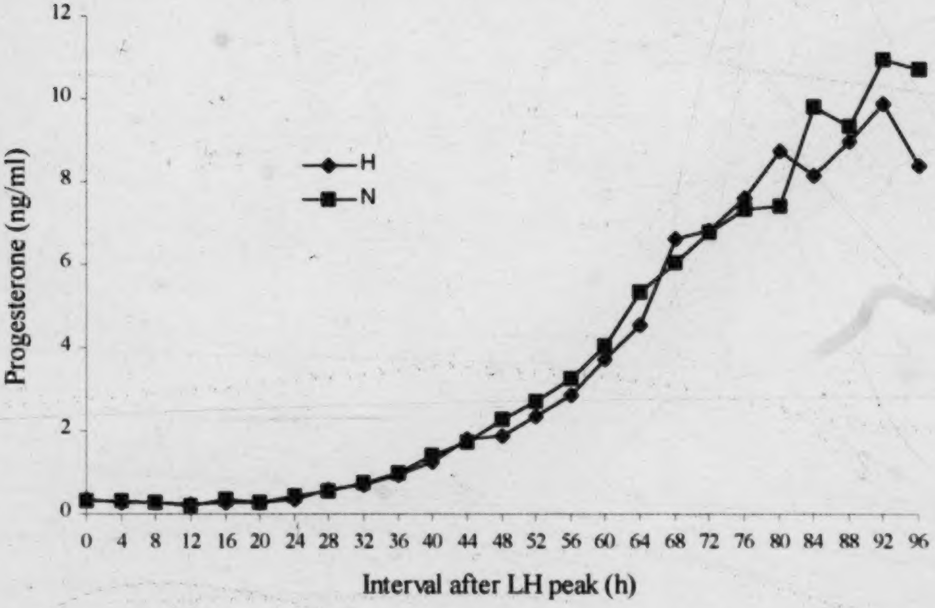
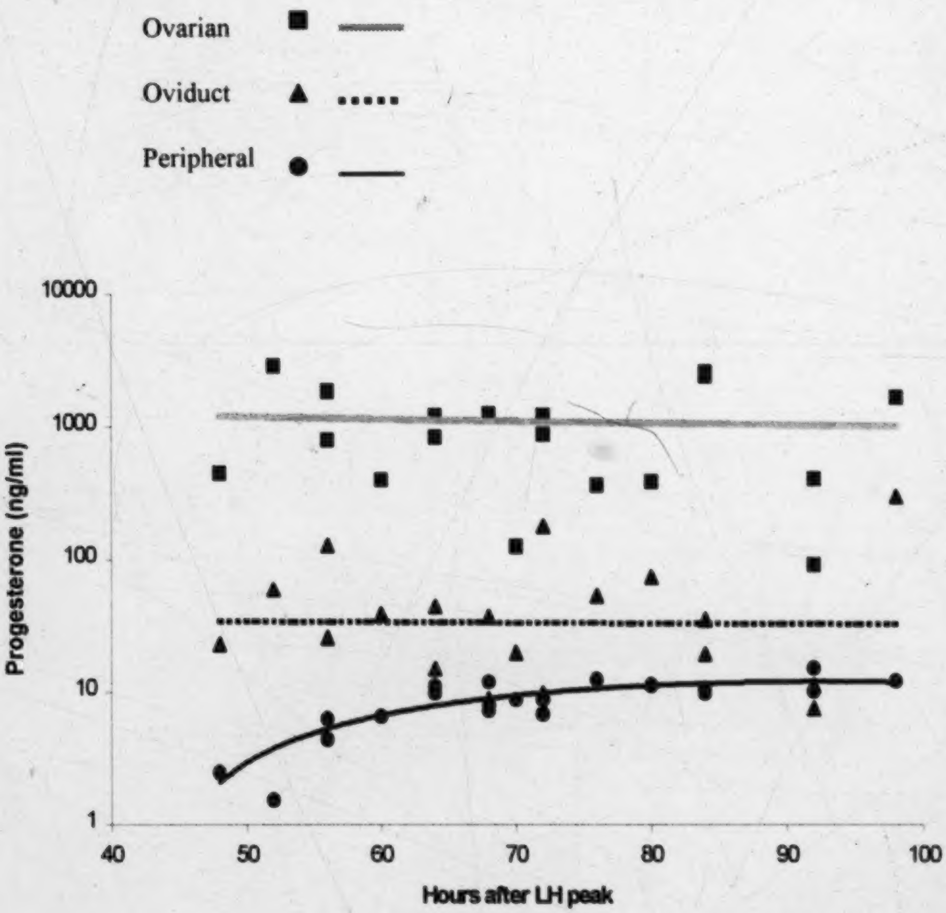


Figure 2.2. Relationships between ovarian, oviductal, and peripheral progesterone concentrations and time after the pre-ovulatory LH peak. Samples were obtained at surgery 72 h after onset of estrus and retrospectively analyzed with respect to time of the pre-ovulatory LH peak determined by RIA. A significant correlation was observed between peripheral progesterone concentrations and time after LH peak (r=0.79, P=0.0001), but not between time after LH peak and oviductal or ovarian progesterone concentrations.



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CHAPTER 3

TEMPORAL PATTERN OF TOTAL PROTEIN, IGF-I AND PORCINE OVIDUCTAL SECRETORY PROTEINS 1-3 (POSP) CONCENTRATIONS IN OVIDUCTAL FLUSHINGS, AND POSP EXPRESSION IN OVIDUCTAL TISSUE AROUND OVULATION IN GILTS

INTRODUCTION

In the gilt, nutrition can affect embryo survival to d 28 of pregnancy (Ashworth, 1991; Pharazyn, 1992; Jindal et al., 1996; Almeida et al., 2000a). In these studies, reduced embryonic survival was associated with lowered progesterone concentrations on d 1 to 3 of pregnancy, and exogenous progesterone treatment immediately after ovulation restored embryo survival (Ashworth, 1991; Jindal et al., 1997). Nutritional effects on embryonic survival may also be related to alterations in follicular development, with respect to maturity and steroidogenic capabilities of the follicle. These effects on follicular development may affect the rate of lutenization and progesterone production of the newly formed corpora lutea (Foxcroft, 1997). As a result, the timing of the estradiol peak, and the increase in progesterone concentrations after ovulation may have significant impacts on embryonic survival (Ashworth et al., 1991; Blair et al., 1994; Soede et al., 1994; Jindal et al., 1997). Both the oviductal and uterine environments are affected by ovarian steroids, and therefore, are implicated in mediating embryonic survival. However, since the uterus is only exposed to peripheral levels of steroids in the pig (Pharazyn et al., 1991; Chapter 2), and progesterone is rising in the oviductal circulation in advance of a rise in the peripheral circulation (Chapter 2), we suspect that the oviduct will be more sensitive to changes in ovarian steroids during the peri-ovulatory period. Oviduct function is regulated by local ovarian steroid concentrations because of the presence of the counter-current multiplier system (see review by Krzymowski et al, 1990; Chapter 1, Section D). In addition, many secreted proteins in the oviduct are estrogen-induced (see reviews by Gandolfi, 1995; Nancarrow and Hill, 1995; Buhi et al., 1997), and therefore nutritional effects on oviduct function may occur during pre-ovulatory follicular development.

The role of the mammalian oviduct in fertilization and embryo development has yet to be fully elucidated. However, co-culture with oviductal cells and fluid has beneficial effects on gamete maturation (Dubuc and Sirard, 1995; Kim et al., 1996), fertilization (Nagai and Moor, 1990), and early embryonic development (Gandolfi and Moor, 1987), suggesting that secretions from oviduct cells play an active role in fertilization and embryonic viability. Our long-term objectives, therefore, are to elucidate how the oviduct is involved in nutritional paradigms that affect embryonic survival. In order to effectively do this, the current study was designed to develop the techniques to assess the quality of the oviductal environment in relation to steroid-responsive proteins secreted by the oviduct during estrus in the pig.

We chose Porcine Oviductal Secretory Proteins 1-3 (POSP) as markers because they are synthesized by the oviduct in response to estrogen (Buhi et al., 1992; Buhi et al., 1996), and become associated with the oocyte and early developing embryo (Buhi et al., 1993). Counterparts to this protein in other species are postulated as having a role in fertilization (Schmidt et al., 1997; Martus et al., 1998). Also, Wallenhaupt et al. (1996), purified the 97 kDa glycoprotein from pig oviductal fluid, and found that in vitro fertilized oocytes had an increased rate of protein synthesis at the 4-cell stage when incubated with this protein.

IGF-I was also chosen as another marker of the quality of the oviductal environment because it is present in highest concentrations in oviduct fluid during estrus in the pig (Wiseman et al., 1992). IGF-I is reported to be beneficial to the cleaving embryo in other species by increasing the proportion of embryos developing to the morula and hatched blastocyst stage in vitro (Matsui et al., 1995; Lighten et al., 1998).

Another important objective of this study was to characterize these oviductal fluid proteins temporally during estrus. Although exogenous estrogen administration in ovariectomized gilts demonstrates that POSP is estrogen-dependent (Buhi et al., 1992; Buhi et al., 1996), it is evident that through the peri-estrus period there is a dynamic interaction between decreasing estrogen concentrations and increasing progesterone concentrations. Steroid concentrations are also 10-fold higher at the oviductal level (Hunter et al., 1983; Pharazyn et al., 1991; Chapter 2), and it is very unlikely that this hormonal milieu can be reproduced in steroid-treated, ovariectomized animals. Therefore, we still do not know how the oviductal environment is affected by dynamic changes in endogenous ovarian steroids around the time of ovulation. In addition, no previous study of oviduct fluid components in the pig in vivo has included a simultaneous measure of estradiol and progesterone concentrations during estrus. Furthermore, previous studies have only defined the oviduct environment temporally in relation to onset of estrus. Since ovulation times are variable with respect to onset of estrus in gilts (Almeida et al., 2000b), defining changes in oviduct fluid components with respect to ovulation may be critical.

Therefore, the objectives of this study were to 1) define the temporal changes in the oviduct environment in relation to time of ovulation using ultrasonography; 2) to develop the methodology to quantify POSP mRNA and protein in oviduct fluid and tissue samples, and to measure IGF-I concentrations in oviduct fluid using radioimmunoassay techniques; and 3) to relate changes in oviduct fluid protein and POSP concentration, IGF-I concentrations and POSP mRNA expression, to peripheral concentrations of estradiol, progesterone and the estrogen:progesterone (E:P) ratio.

MATERIALS AND METHODS

Animals

Twenty cyclic Camborough x Canabrid terminal line gilts (Pig Improvement (Canada) Ltd.) used in this experiment were housed at the University of Alberta Swine Research Unit, in barns with a totally controlled environment. They were individually fed twice daily a total of 2.0 X Energy Maintenance requirements of a standard barley-wheat-soybean meal grower diet containing 14.16 MJ DE/kg and 14% CP. Maintenance requirements were calculated within 10 kg increments in body weight, to provide a maintenance energy allowance of 461 kJ DE/kg of metabolic BW. Gilts had ad libitum access to water. All animals were cared for in accordance with CCAC Guidelines with authorization from the Faculty Animal Policy and Welfare Committee. The gilts were randomly assigned to one of five slaughter times around ovulation (Table 1). For all gilts, estrus detection was carried out every 8 h starting at d 18 of the cycle using backpressure testing during fenceline contact with a vasectomized boar. The time of the onset of estrus was determined as the time of occurrence of a standing reflex in the presence of a boar, minus 4 h. Beginning at 8 h after the standing reflex was first observed, the right ovary in each gilt was examined by transcutaneous ultrasonography (Pie Medical Scanner 200, model 41480, Can Medical, Kingston, Ontario), using a 5.0-7.5 MHz multiple scan angle transducer for the presence of preovulatory follicles. Gilts were scanned every 8 h until ovulation was completed. Time of ovulation was defined as the first scanning when no presumptive ovulatory follicles were seen, minus 4 h.

Sample Collection

Between 1 and 2 h before slaughter, heparinized blood samples (3ml) were taken from an ear vein. All blood samples were centrifuged (2200Xg, 4°C) and plasma separated and stored at -30°C until assayed for progesterone and estradiol concentrations. Gilts were transported to a local abattoir between two days before, and two days after, ovulation (see Table 3.1) and slaughtered. Immediately after slaughter, reproductive tracts were removed from each gilt. Right and left sides were identified, and ovaries were examined and confirmed as pre-ovulatory or post-ovulatory. If ovulation had occurred, corpora lutea were counted on both ovaries as a measure of ovulation rate. The oviducts were tied at the uterotubal junction and clamped tight at the infundibulum. The oviducts were dissected free of connective tissue and the rest of the reproductive tract by blunt dissection. A blunted 20g needle attached to a syringe was inserted into the oviduct lumen through the uterotubal junction and the oviduct was then flushed with 5 ml of physiological saline. The

Table 3.1. Definitions and strategy for establishing sampling times.

Sample Time	Number of gilts	Definition/Sample collection	Confirmation
Day –2	5	48 to 24 h prior to ovulation*	At slaughter, follicles were between 5-7 mm
Day –1	3	24 to 0 h prior to ovulation	Ultrasonography performed every 8 hours to ensure that follicles were > 7 mm and pre-ovulatory. Confirmation at slaughter with follicle measurement.
Day 0	5	0 to 24 h after ovulation	Successive ultrasonography and presence of corpora hemorraghica at slaughter.
Day 1	3	24 to 48 h after ovulation	Successive ultrasonography and presence of newly formed corpora lutea at slaughter.
Day 2	4	48 to 72 h after ovulation	Successive ultrasonography and presence of corpora lutea at slaughter.

* Estimated on the basis of a previous study using ultrasonography which showed that gilts of this genotype ovulate on average 45 hours after onset of estrus. (Almeida et al., 2000b)

flushings were immediately placed on ice for transportation and then stored at -30°C until further analysis. Midsections of ampulla and isthmus were dissected from the oviduct, snap frozen in liquid nitrogen, and subsequently stored at –80°C.

Radioimmunoassays

Plasma progesterone concentrations were determined in duplicate using an established radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corporation, U.S.A.), previously validated for use with porcine plasma without extraction (Mao and Foxcroft, 1998). The sensitivity of the single assay, defined as 86.4 % of total binding, was 0.097 ng/ml and the intra-assay CV was 5.7 %.

Estradiol-17ß was extracted from 1 ml plasma samples by the addition of 5 ml diethyl ether (VWR Canlab, Mississauga, ON, Canada) and vortexing for eight 1-min pulses. Extraction tubes were then placed in a liquid nitrogen/ methanol bath and the aqueous layer was allowed to freeze. The supernatant was then poured off and dried under vacuum. Samples were reconstituted with assay buffer to manufacturers specifications and determined in duplicate in a single radioimmunoassay using a double antibody kit from Diagnostics Products Corporation (Los Angeles, California, USA), previously validated for use with porcine plasma (Yang et al., 2000), with modifications. The second antibody was diluted 3-fold to increase sensitivity. Recovery of radiolabelled hormone was 83.7 ± 11.7 %, and samples were not corrected for recovery. The assay sensitivity for the single assay run, defined as 84.0 % of total binding, was 0.3206 pg/tube and the intra-assay CV was 7.2 %.

IGF-I concentration in peripheral plasma was determined using the homologous double antibody radioimmunoassay described previously (Cosgrove et al., 1992). The anti-human IGF-I antiserum (product name AFP4892898, obtained from Dr. AF Parlow through the NIDDK's National Hormone and Pituitary Program), was used at a 1/654,000 final dilution, resulting in 38% specific binding. The single assay run had an intra-assay coefficient of variance of 6.9% and the sensitivity, defined as 93.7% of total bound, was 0.015 ng/tube. Recovery efficiency was 86.6 ± 2.5% and samples were not corrected for recovery.

IGF-I concentration in oviduct flushings was determined using the same assay with modifications to the extraction procedure. To concentrate the samples sufficiently for detection, 0.5 ml or 1 ml of oviduct flushings were lyophilized, reconstituted in 0.4 ml assay buffer (phosphate buffered saline containing 0.1% (w/v) gelatin, pH 7.0), and taken to extraction to remove bound proteins. Finally, 300 µl of neutralized acid extract was taken into the assay to ensure that the binding produced by experimental samples fell on the linear portion of the standard curve. Diluted oviduct flushings samples showed parallelism to the standard curve. Recovery efficiency was 98.6 ± 4.4%, and samples were not corrected for recovery. The assay sensitivity for the single assay run, defined as 98.8% binding, was 0.00195 ng/tube and the intra-assay CV was 11.46 %.

Oviduct fluid protein determination and Western blotting

Oviduct flushings were thawed on ice, the volume recorded, centrifuged (2200Xg, 4°C) for 10 min, and then dialyzed against 10mM Tris buffer (4L, 4°C) for 24 h with one change. Samples were then assayed for protein determination using BCA assay (Sigma) and freeze dried in 50ug aliquots. 5 µg of total protein from experimental samples, and from a positive (pooled oviduct fluid collected at estrus) and negative (pooled oviduct fluid collected at d 28 of pregnancy) control, were loaded onto 1.5mm thick, 4%-10%(w/v) gradient SDS-PAGE gels in duplicate. After electrophoresis, one gel was silver stained to correct for protein loading and the other was transferred onto an ECL-Hybond (Amersham Life Sciences, Buckinghamshire, England) nitrocellulose membrane overnight at 325mA using a Transblot apparatus (Bio-Rad Labs, Richmond, CA). Non-specific binding was blocked in 5%(w/v) BSA in TBS-T for 1 h, and blots were incubated with polyclonal antibody raised in rabbits against POSP 1-3 (a gift from Dr. Buhi, University of Florida) at a 1:8000 dilution for 1 h. This antibody has been reported to be specific for POSP 1-3 in pigs (Buhi et al. 1993). Buhi et al. (1996) proposed that POSP 1, 2 and 3 are derived from the same protein and the same gene and we therefore, will refer to POSP 1, 2, and 3 collectively as POSP. Horseradish-peroxidase conjugated goat anti-rabbit IgG was then used as the secondary antibody and incubated for 20 min with the blots. Immunoreactive proteins were detected using the Western blotting ECL system as directed by the manufacturer (cat no. RPN2206; Amersham Life Sciences, Buckinghamshire, England). Protein bands for POSP 1, and 2-3 were quantified using densitometric techniques (Molecular Analyst v2.01, Bio-Rad Labs, Richmond, CA), values were grouped together, and collectively termed POSP. POSP was expressed as a proportion of the positive control sample density and corrected for protein loading on the corresponding silver-stained gel. The control sample was run with each blot to allow for standardization of blots and comparison across gels. The average densitometric value of duplicate samples was used for statistical analysis.

POSP mRNA expression analysis

A subset of 14 animals (n= 3, 3, 3, 2, and 3 gilts, for Days –2 to 2, respectively), selected on the basis of plasma progesterone and estradiol concentrations, were used for analysis of steady-state levels of POSP mRNA.

Total RNA was extracted from ampulla and isthmic sections of oviducts using TRIzol Reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). The RNA was quantified by spectrophotometric absorbance at 260 nm, then aliquoted and stored at -80°C. RNA integrity was assessed by observing the 28S and 18S ribosomal bands after electrophoresis of one aliquot on a 1% (w/v) formaldehyde agarose gel followed by ethidium bromide staining.

Northern blots were prepared by subjecting 20 μg of total RNA to electrophoresis in 1% (w/v) agarose gels in duplicate under denaturing conditions with formaldehyde (Yuan et al., 1996). mRNA was then transferred to Hybond-XL nylon membrane (Amersham Pharmacia Biotech, Baie d'Urfé, QC) overnight and then baked at 80°C for 2 h under vacuum. Standard RNA size markers (Life

Technologies, Gaithersburg, MD) were used to estimate the size of mRNA transcripts detected on Northern blots.

Blots were pre-hybridized for a minimum of 3 h at 60°C in 5x SCC (single-strength SCC= 150 mM sodium chloride, 15 mM citric acid), 100 µg/ml sheared salmon sperm DNA and 5x Denhardts solution (100x Denhardts = 2% (w/v) bovine serum albumin, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone). Blots were then hybridized overnight in fresh prehybridization solution using a 0.8 kb cDNA for POSP (obtained from Dr. W.C. Buhi, Univeristy of Florida, Gainsville, FL), to assess steady state mRNA levels. The message detected by the probe is for POSP 1, 2, and 3 as they are products of the same gene (Buhi et al., 1996). The cDNA fragment was labeled with [32P]-dATP (Amersham Pharmacia Biotech, Baie d'Urfé, QC) using a Random Primed DNA labeling kit according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). Unincorporated nucleotides were removed using G-50 Sephadex Nick Columns (Amersham Pharmacia Biotech, Baie d'Urfé, QC). After hybridization, the blots were washed twice (15 min each time) with 2x SSC /0.1 % (w/v) SDS at 35°C, followed by two washes (15 min each time) with 1x SSC/ 0.1% SDS (w/v), and finally by one 20 min wash in 0.2x SSC/0.1% SDS (w/v) at 68°C. Blots were exposed to BioMAX MS film (Eastman Kodak, Rochester, NY) for 24 h at -80°C with 2 intensifying screens. All RNA data were normalized for loading and are expressed as POSP abundance /28S in arbitrary units.

Statistical analysis

Normal distribution of data was checked by the UNIVARIATE NORMAL procedure of SAS (1990), and plasma progesterone and estradiol concentrations, E:P ratio, and IGF-I concentrations in oviduct flushings were log transformed to achieve normal distribution. Plasma progesterone, estradiol and IGF-I concentrations were analyzed using the GLM procedure of SAS: the model used was a simple ANOVA, which included Day relative to ovulation as the independent variable and variance across animals as the error term. The volume recovered, total protein, protein concentration, IGF-I concentration and POSP values in oviduct flushings were analyzed by the GLM procedure of SAS (1990), using day and gilt within Day relative to ovulation as the independent variables and variation across animals within Day as the error term. Multiple comparisons of means were analyzed using the method of Tukey's Honestly Significant Difference. If unequal sample sizes existed, Least Squares Difference test was used to compare differences of means. Both tests were only performed if the statistical model and Day were both significant ($P<0.05$). All correlations were carried out using linear regression analysis (SAS 1990). The data are presented as LSM (± SE of LSM).

RESULTS

Plasma progesterone, estradiol, and IGF-I concentrations, and E:P Ratio

Day significantly affected both progesterone concentrations ($P=0.0001$) and E:P Ratio ($P=0.0001$), but not estradiol concentrations (Figure 3.1). IGF-I concentrations in plasma did not change between Day –2 (164.3 ± 15.0 ng/ml), Day 0 (143.51 ± 11.6 ng/ml), Day 1 (124.2 ± 26.0 ng/ml), and Day 2 (132.0 ± 13.0 ng/ml).

Oviduct flushings

Total volume recovered did not change with time (4.92 ± 0.07 ml, 4.58 ± 0.10 ml, 4.77 ± 0.07 ml, 4.62 ± 0.10 ml and 4.73 ± 0.08 ml for Day –2 to Day 2, respectively). However, total protein concentration ($P=0.012$) and total protein content ($P=0.008$) in oviduct flushings were different across days (Figure 3.2). The differences across days were evident between pre (Day –2 and Day –1) ovulation and post (Days 0, 1, and 2) ovulation. Total protein concentrations were correlated to

E:P ratio (r=0.48, P=0.05) and negatively correlated to progesterone concentrations (r=-0.44, P=0.05) in peripheral plasma.

IGF-I concentrations in oviduct flushings were higher (P=0.0001) at Day –2, while Day –1 was intermediate, compared to Day 0, Day 1 and Day 2 (Figure 3.2). IGF-I concentrations were also strongly correlated to E:P ratio in peripheral plasma (r=0.70, P=0.002). Total protein concentrations were correlated to IGF-I concentrations in oviduct flushings (r=0.46, p=0.004), and IGF-I concentrations and total protein content were also correlated (r=0.51, P=0.001).

POSP protein in oviduct flushings

The Western blotting technique resulted in immunoreactive protein bands migrating at 88 kDa and 115 kDa (Figure 3.3A). These bands correspond to the 85kDA POSP 1, and the 100kDA POSP 2-3 that migrate as one 115kDa band on a 1D SDS-PAGE gel as described by Buhi et al. (1989), and are collectively referred to as POSP. Specific binding of the primary and secondary antibody was confirmed by the lack of immunoreactive staining in the negative control pool (Figure 3.3B). POSP abundance (arbitrary units per μg of total protein) as measured by densitometry was lower (P=0.001) in Day 2 oviductal flushings as compared to other days (Figure 3.4). However, when expressed as a concentration in oviduct flushings (POSP abundance X total protein concentration), there was an intermediate effect of Day 0 and Day 1 on POSP concentration compared to Days 2, and Days –2 and –1 (Figure 3.4).

Plasma progesterone concentrations were negatively correlated with POSP abundance (r= -0.48, P=0.04) and POSP concentration (r= -0.67, P=0.002) in oviduct flushings. Also, POSP concentrations were positively correlated with the E:P ratio (r=0.624, P=0.009).

IGF-I concentrations in oviduct flushings were positively correlated with POSP abundance (r=0.47, P=0.004) and with POSP concentration (r=0.76, P=0.0001). POSP abundance was negatively correlated with total protein concentration in flushings (r=-0.65, P=0.0001) whereas POSP concentration in flushings was positively correlated with total protein concentration (r=0.75, P=0.0001).

POSP mRNA analysis

Northern blot analysis of POSP revealed hybridization bands at approximately 2.25 Kb (Figure 3.5A). There was a significant effect (P=0.01) of Day on POSP mRNA abundance, as mRNA expression had almost completely disappeared by Day 1 and was gone by Day 2 (Figure 3.5B). POSP mRNA abundance was highly correlated with the E:P ratio (r=0.75, P=0.005) and inversely related to plasma progesterone concentrations(r= -0.73, P=0.004). POSP mRNA abundance was not related to POSP protein abundance in flushings.

DISCUSSION

As the oviduct environment is thought to have an active role in gamete maturation and transport, fertilization, and in early embryonic development, research has focused on the specific oviductal secretions during the peri-estrus period and their possible role in embryo viability. Previous studies have looked at components of the oviduct fluid and de novo protein synthesis in relation to the onset of estrus; however, the time interval between ovulation and onset of estrus is highly variable in gilts (Almeida et al., 2000b) and sows (Soede et al., 1995). In this study, we provide evidence that the time of ovulation is critical in characterizing the oviduct environment during estrus, because ovulation changes the hormonal profiles in the oviductal circulation. This is especially important when future attempts will be made to elucidate nutritional effects in the oviductal environment that may contribute to embryonic loss. To our knowledge, this study is the first to characterize oviduct fluid components in relation to ovulation, and to relate these components to plasma estradiol and progesterone concentrations.

The peri-ovulatory profile of steroids showed that peripheral estradiol concentrations were dropping from 48 h before ovulation, and remained low after ovulation, whereas peripheral progesterone concentrations were low until ovulation (Day 0) and then rose substantially after that time. As a consequence, the E:P ratio changed throughout the peri-ovulatory period, getting progressively smaller from Day –2 to Day 2. The changes in peripheral estradiol and progesterone concentrations and the E:P ratio throughout the peri-estrus period demonstrate the dynamic hormonal milieu to which the oviduct is exposed. Although hormone concentrations were not measured in the oviductal circulation, we know that they are much higher (Hunter et al., 1983; Pharazyn et al., 1991; Chapter 2) compared to peripheral concentrations because of the sub-ovarian counter-current multiplier system (Krzymowski et al., 1990). Furthermore, we were able to show that concentrations of progesterone were already high 48 hours after the LH peak in the oviductal veins, but were still rising in the peripheral circulation (Chapter 2). Also, Eiler and Nalbandov (1977) showed that progesterone concentrations were rising 16 h in advance of ovulation in the utero-ovarian vein, suggesting that the oviduct is responsive to local concentrations of ovarian steroids that are not reflected concurrently in the peripheral circulation. By responding rapidly to changes in ovarian steroids, the oviduct can provide an optimal environment for the gametes or developing embryo. However, despite this knowledge we chose to examine peripheral levels of estradiol and progesterone so that the results from this study can be put into context with other studies looking at the oviduct and embryonic survival. It is critical to elucidate the changes in the oviductal environment around ovulation to gain insight in the role of the oviduct in embryonic survival, and the most important step is to report the data with respect to time of ovulation.

Higher progesterone concentrations after ovulation have been linked to increased embryonic survival in pigs (Ashworth et al., 1991; Pharazyn, 1992; Jindal et al., 1996; Almeida et al., 2000a). The highly fertile Meishan sow also exhibits a faster rise in plasma progesterone after the LH surge compared to commercial white breeds (Hunter et al., 1996). However, conflicting studies exist, showing that if progesterone is artificially increased above normal concentrations by food deprivation, the cleavage rate of embryos is reduced in sows (Mburu et al., 1998). Also, in contrast to positive results in feed-restricted gilts (Ashworth et al., 1991; Jindal et al., 1997), exogenous progesterone treatment in weaned sows resulted in increased embryonic loss (Mao and Foxcroft, 1998). In both studies, these effects were thought to be either due to accelerated transport of the embryo through the oviduct, or lower fertilization due to inappropriate timing of sperm transport in the oviduct. In addition, cows that are given exogenous steroids to synchronize cycles have also been shown to have decreased fertility (Savio et al., 1993), possibly resulting from changes in the patterns of oviduct protein secretion (Binelli et al., 1999).

Circulating progesterone and estrogen concentrations can affect oviductal vascularity (Ford, 1982), contractility (Battalia and Yanagimachi, 1980), and fluid formation and protein synthesis (Lippes et al., 1981; Buhi et al., 1989; Wiseman et al., 1992). Also, many proteins synthesized by the oviduct are estrogen-dependent (Buhi et al., 1992). Therefore, it is possible that priming of the oviduct with appropriate concentrations of estradiol during follicular development is also as important as the timing of the rise in plasma progesterone.

Feed restriction of gilts during the estrous cycle (Almeida et al., 2000a) and sows during lactation (Zak et al., 1997), has also been shown to reduce embryonic survival. These studies suggested that previous feed restriction adversely affected follicular maturation, and impacted steroidogenic ability of the follicle before ovulation and subsequent lutenization. Although the quality of the oocyte may also have an impact on embryonic survival (reviewed by Foxcroft, 1997), nutritionally-induced changes in peri-estrus hormone profiles (Almeida, 2000), could alter the steroid priming of the oviduct and thus change the oviduct environment. Blair et al. (1994) and Soede et al. (1994) showed that higher embryonic survival in sows was associated with changes in the timing of peak estradiol and LH surge in relation to onset of estrus, and a short interval between peak estradiol and rising progesterone concentrations during the peri-estrus period. From the current study, we know that oviduct secretion is associated with estrogen and progesterone concentrations and the E:P ratio, providing evidence for a link between alterations in hormone profiles in the peri-estrous period and the timing of oviduct secretions. As a consequence, the oviduct may not provide an optimal environment to the gametes and the developing embryo and thus contribute to the asynchrony of the embryos and uterus (reviewed by Pope, 1988), leading to lower embryonic survival.

In this study, there was a negative relationship between total protein concentration and progesterone concentrations, which is initially in contrast to the study by Lippes et al. (1981), who found that protein concentrations in human Fallopian tube fluid was inversely correlated with plasma estradiol concentrations. However, they argue that the synthesis of fluid immediately before ovulation was enough to mask maximal protein production by the oviduct, because total protein content (protein concentration X fluid volume) in fluid did correlate with plasma estradiol concentrations. Buhi et al. (1990) have shown that estrus porcine oviductal tissue has the highest levels of de novo protein synthesis and secretion, which would also agree with our findings. However, this study was able to further define total protein concentrations and total protein content in porcine oviductal flushings in relation to time of ovulation. The highest total protein concentrations and total protein content were present in Day –2 and Day –1, and then sharply declined on Day 0, and remained at those levels. The sharp decline in total protein content and concentrations are coincident with the rise of progesterone concentrations in the oviductal circulation, and not the peripheral rise (Eiler and Nalbandov, 1977; Hunter et al., 1983; Chapter 2), suggesting that oviduct protein secretion is responsive to steroid concentrations at the oviductal level. The temporal pattern of total protein concentrations and content are similar to the observed decline of IGF-I concentrations and POSP concentrations in oviduct flushings in this study, providing further evidence of regulation by ovarian steroids at concentrations present in the oviductal circulation.

The importance of this study also extends into the characterization of POSP mRNA and the secreted POSP proteins in relation to ovulation, further defining the temporal pattern of synthesis and secretion of POSP. This is important in elucidating the role of the oviduct in embryonic survival, because of the potential roles of this glycoprotein in early embryonic development (Buhi et al., 1997). Nutritionally induced alterations in hormone profiles may change the timing of POSP synthesis and secretion and the concentrations present in oviduct fluid, thus possibly influencing early embryonic development. By characterizing the temporal pattern of POSP synthesis and secretion in untreated pigs, we are able to better interpret changes in the oviduct environment in response to nutritional manipulation in future studies.

POSP mRNA was present until 24 h after ovulation, decreased substantially by Day 1 and disappeared by Day 2 after ovulation. These results are consistent with data from the only other study examining POSP mRNA expression. Buhi et al. (1996) reported that expression was high at d 0 of the cycle (onset of estrus) which corresponds to our Day –2, and disappears by d 2 of the cycle, which corresponds to our Day 0 or Day 1. However, considering our recent data on the variation in estrus to ovulation interval in gilts (Almeida et al., 2000b), our results provide a more accurate description of POSP mRNA expression during the peri-estrus period. The relationships established between steroid concentrations and POSP mRNA expression are consistent with evidence that POSP expression is estrogen-dependent (Buhi et al., 1996). Although POSP mRNA was still present when peripheral progesterone concentrations were rising, it is possible that the mRNA was fairly stable, as we analyzed steady-state expression only. Also, the disappearance of POSP mRNA preceded the disappearance of POSP protein in the oviduct flushings by 24 h. This may be due to incomplete turnover of oviduct fluid during this time, or active secretion of already synthesized POSP from the epithelial cells in the oviduct.

IGF-I has previously been characterized in oviduct fluid in the pig (Wiseman et al, 1992). However, although these authors found that IGF-I content (concentration X volume) was highest at estrus, there were no significant differences between IGF-I concentrations at estrus (27.6 ± 6.3 ng/ml) and non-estrus periods (24.7 ± 6.3 ng/ml). This differs from our findings, as IGF-I concentrations in this study were very similar in magnitude (range 9.1 to 29.4 ng/ml) to those reported by Wiseman et al. (1992), but we found differences in IGF-I concentrations between Day –2, Day –1 and Day 0 to 2. This is likely due to the fact that Wiseman et al. (1992) pooled data from d 0 to 3 of the estrous cycle and as a consequence, they reported large variability in IGF-I concentration means and estradiol peaks from gilts used in their study, in relation to onset of estrus. Our ability to demonstrate temporal differences in IGF-I concentrations in oviduct flushings during estrus is mainly due to an interpretation of our data with respect to time of ovulation. This discrepancy between studies demonstrates the importance of characterizing oviduct fluid components in relation to time of ovulation.

An interesting finding of this study, consistent with the findings of Wiseman et al. (1992), is that IGF-I concentrations in the oviduct flushings were not correlated to plasma IGF-I concentrations. IGF-I concentrations in plasma did not change through the peri-ovulatory period, whilst IGF-I concentrations in the oviduct flushings were higher before ovulation than after ovulation, and were strongly associated with the E:P ratio. Also, as we observed that IGF-I concentrations were strongly correlated with other fluid components such as total protein and total protein concentration, steroid driven oviduct fluid synthesis appears to be the major determinant of IGF-I concentrations in oviduct fluid. If the majority of IGF-I present in the oviduct flushings is serum derived, it is very likely that the fluid synthesis dynamics around ovulation impact the concentration of IGF-I that is transferred into fluid. However, cultured estrous oviductal epithelial cells secrete IGF-I (Wiseman et al., 1992; Xia et al., 1996; Winger et al., 1997), and estrogen and progesterone modulate IGF-I mRNA expression in the porcine uterus (Simmen et al., 1990). This suggests that oviduct synthesis of IGF-I can contribute to IGF-I concentrations in oviduct fluid, and provides an explanation as to why no relationship was observed between plasma and oviduct fluid. Wiseman et al. (1992) also suggested that oviduct synthesis is the main contributor of IGF-I content in the fluid, because the plasma would have to contain larger amounts of IGF-I during estrus in order to compensate for larger oviduct fluid volumes and a higher IGF-I content at that time. In contrast, IGF-I secretion by cultured human Fallopian tube epithelial cells was undetectable, and Lee et al. (1992) reported very low secretion of IGF-I by cultured porcine oviductal epithelial cells.

Nevertheless, the increase in IGF-I concentrations in oviduct flushings during the peri-ovulatory period suggests that IGF-I may be important to the gametes, developing embryo or act on oviductal epithelial cells. The actions of IGF-I on cell proliferation, differentiation and cell function are well established (see review by Jones and Clemmons, 1995) and its role in early embryonic development has been discussed (Heyner et al., 1993; Schultz et al., 1993; Kaye, 1997; Heyner, 1997). Beneficial effects of IGF-I on the in vitro development of cleaving embryos and blastocysts have been reported (Matsui et al., 1995; Lighten et al., 1998), but we do not know how early the IGF-I receptor is expressed on porcine embryos. Therefore, the potential effect of IGF-I on porcine embryos in the cleavage stage remains to be determined.

Although we have only examined POSP and IGF-I in this study, many other growth factors, hormones and proteins have been identified in the porcine oviduct that are also thought to play a role in optimal oviduct function and embryonic development (see review by Buhi et al., 1997). However, our findings show that like POSP and IGF-I, ovarian hormones and the dynamics of oviduct fluid synthesis could also affect these other factors during the peri-ovulatory period and contribute to the role of the oviduct in embryo survival.

In conclusion, we have shown that the peri-ovulatory profile of oviduct fluid components is very dynamic and responsive to estradiol and progesterone concentrations, and the E:P ratio. However, total protein content and concentration, and POSP and IGF-I concentrations correspond closely with presumed oviductal levels of steroids, rather than the peripheral concentrations of steroids. This study has also provided insight into the profiles of certain fluid components that have been implicated in embryonic development, and allows for better interpretation of the impact of the oviduct environment in embryo survival studies. Our data emphasizes the need to look at the timing of these events with respect to the time of ovulation rather than the time of the onset of estrus, and demonstrate the effective use of transcutaneous ultrasonography to achieve this objective.

Figure 3.1. Least squares means (± SEM) of peripheral plasma estradiol-17ß and progesterone concentrations and the estrogen to progesterone (E:P) ratio across day in relation to time of ovulation. Least squares means were based on a total of 20 gilts (N= 5, 3, 5, 3, and 4 gilts, for Days –2 to 2, respectively).

[a,b,c,d,w,x,y,z] Means with different superscripts between days, within hormone, are different ($P<0.05$).

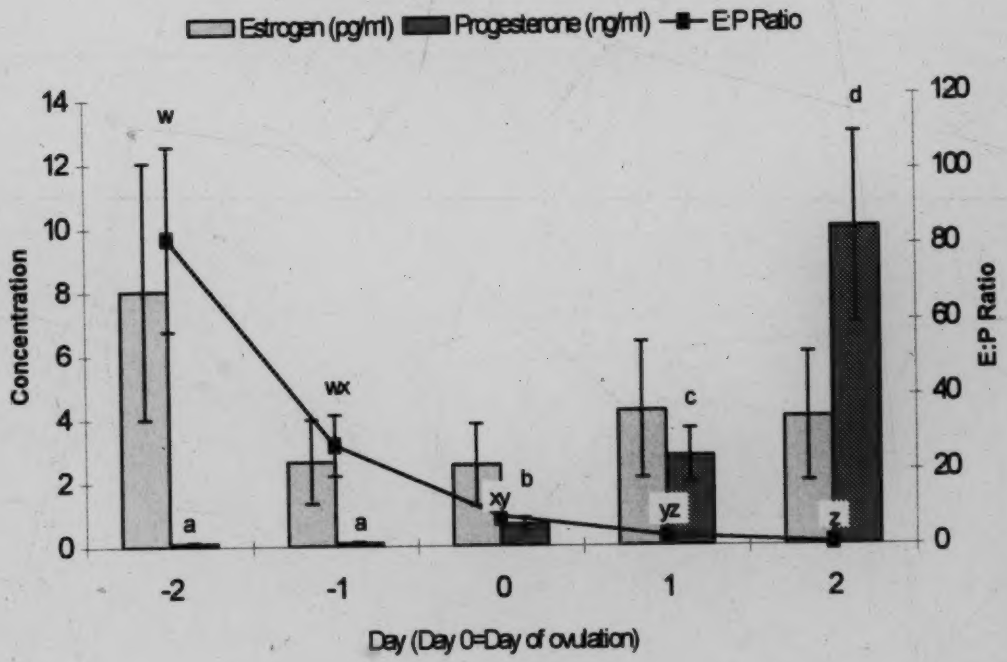


Figure 3.2. Least squares means (± SE of LSM) of total protein concentration, total protein content, and IGF-I concentrations in oviduct flushings in relation to time before or after ovulation. Least squares means were based on a total of 20 gilts (N= 5, 3, 5, 3, and 4 gilts, for Days –2 to 2, respectively).

* Means are different from their respective Day 2 means (P<0.05).

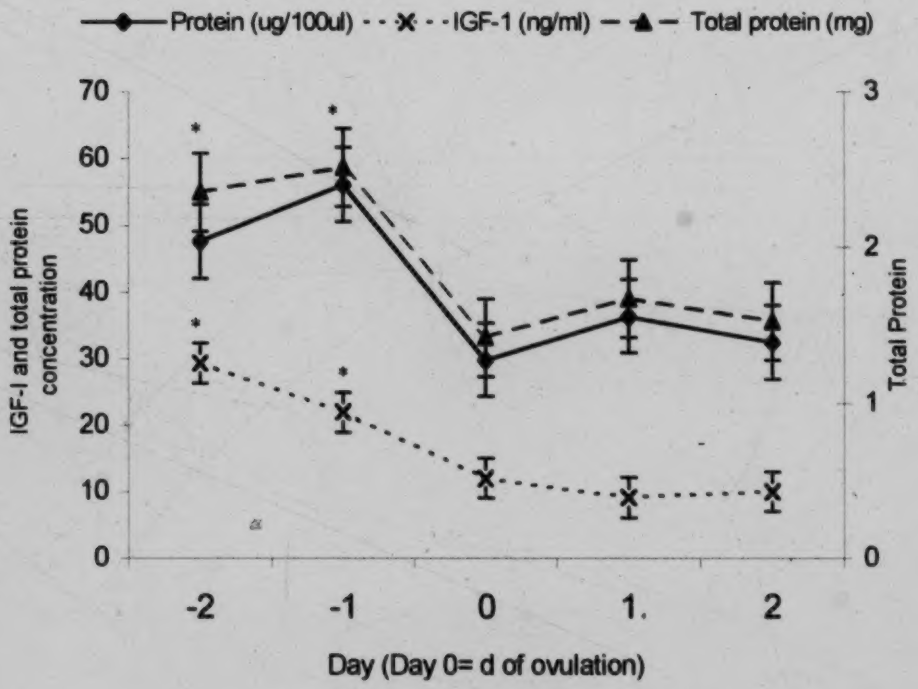


Figure 3.3. A) Representative Western blot of POSP 1-3 (POSP) in oviduct flushings of five gilts slaughtered between day –2 to 2 with respect to day of ovulation. Immunoreactive bands were detected migrating at approximately 88 kDa and 115 kDa. The 88 kDa band corresponds to POSP 1, and POSP 2-3 are the 115kDa band as they migrate together on 1-D SDS-PAGE gels. Positive control (+) is indicated. **B)** Representative Western Blot of Positive (lane 1) and Negative (lane 2) Control pools. Negative Control Pool is pooled oviduct flushings collected from Day 28 pregnant gilts. **C)** Corresponding silver-stained SDS-PAGE gel to Western blot shown in panel A, lanes from left to right are, marker lane, blank, and then the same as panel A.

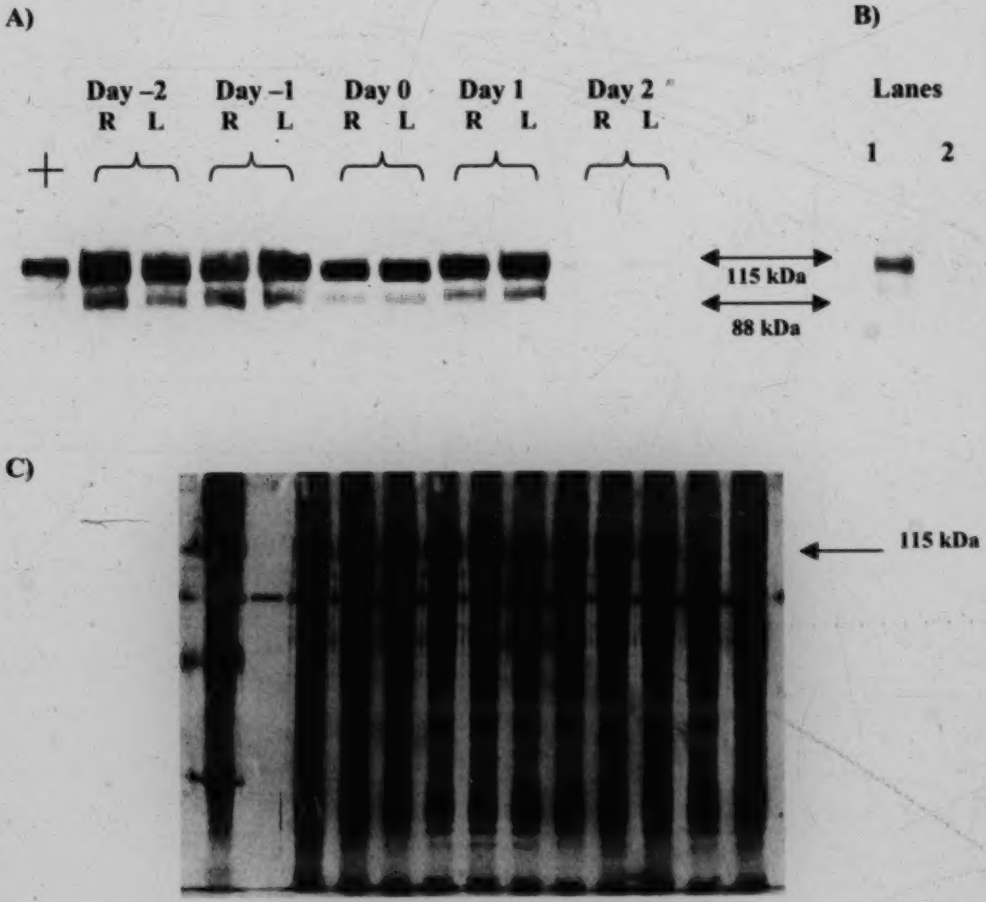


Figure 3.4. Least squares means (± SE of LSM) of POSP abundance per μg total protein recovered (u) and POSP concentration /ml oviduct flushings (n) across time in relation to ovulation. Bands corresponding to POSP 1, 2, and 3 (collectively termed POSP) were quantified using densitometric techniques and expressed as a proportion of the corresponding positive control bands on that blot, in arbitrary units. The POSP protein means (n = 5, 3, 5, 3, and 4 gilts for Days –2 to 2, respectively) were expressed as POSP abundance (arbitrary units per μg total protein) or POSP concentration (POSP abundance X total protein concentration).

[a,b,x,y] Means within measurement with a different letter subscript differ (P<0.05).

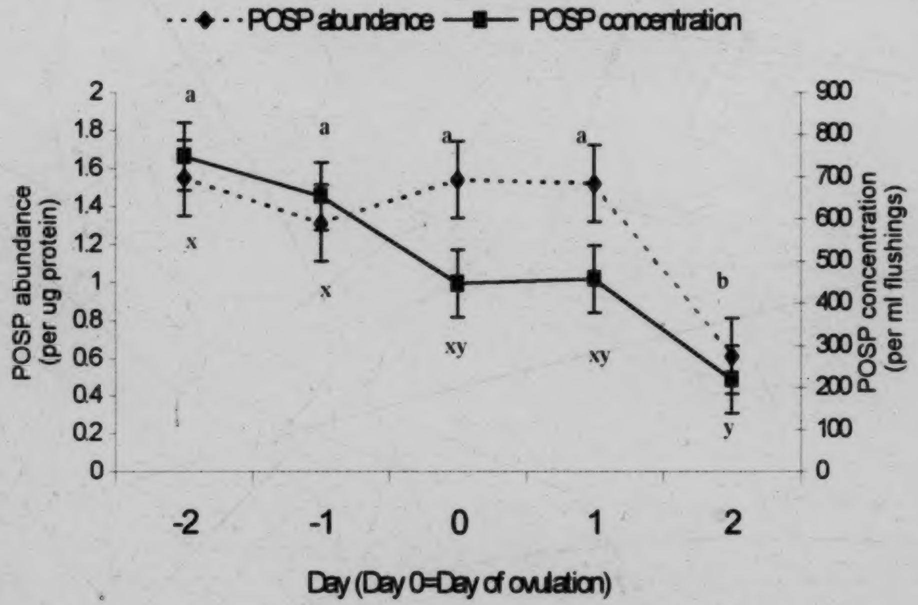
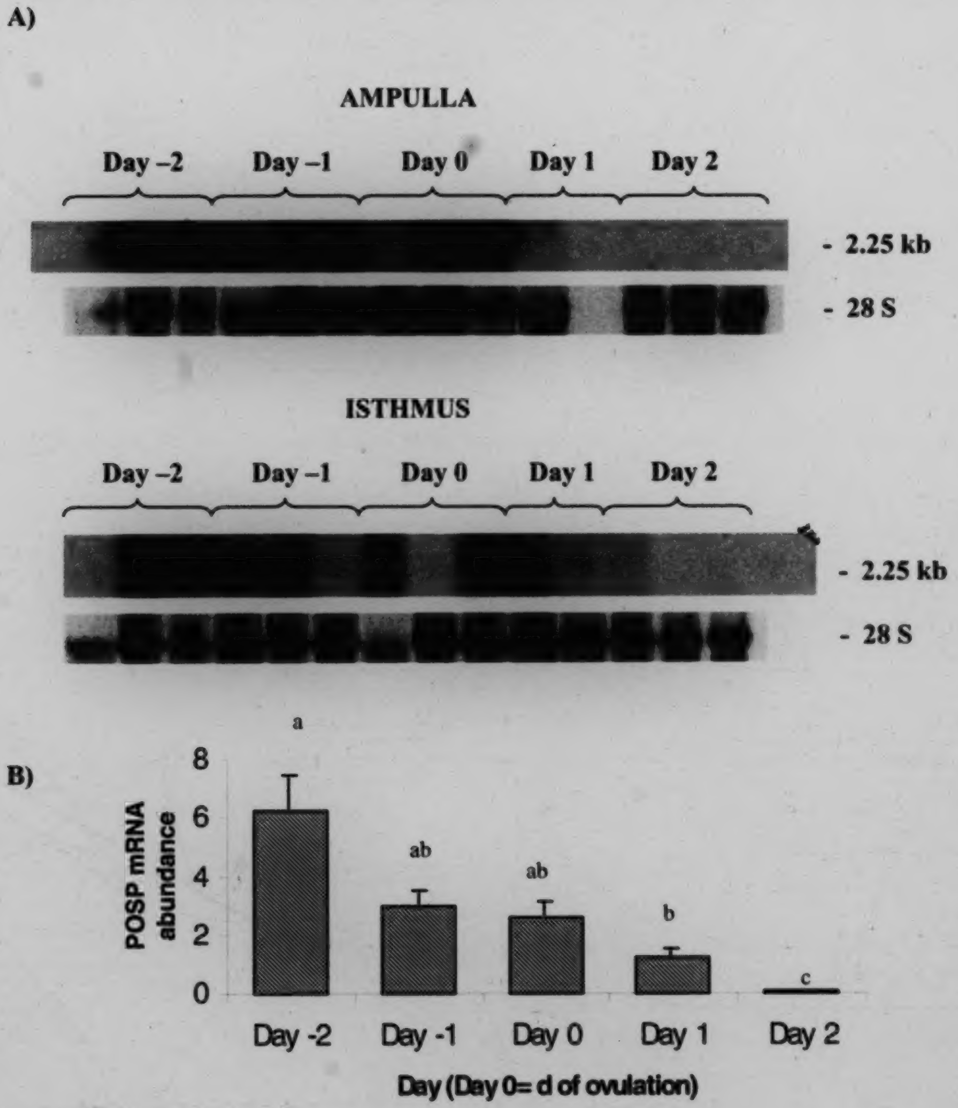


Figure 3.5. A) Northern blot of POSP mRNA from 14 gilts across days (n=3 gilts for Days –2, -1, 0, and 2, and n=2 gilts for Day 1) relative to ovulation. Ampulla and isthmus samples are shown separately. 28s mRNA bands on corresponding gel are shown below each panel. **B)** POSP mRNA abundance in relation to day (d 0= d of ovulation). ^{abc} Means with different letter subscript differ (P<0.05).



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CHAPTER 4

UNILATERAL OVARIECTOMY IN THE GILT AFFECTS PORCINE OVIDUCTAL SECRETORY PROTEIN 1-3 (POSP) SECRETION AND INSULIN-LIKE GROWTH FACTOR-1 CONCENTRATIONS IN OVIDUCT FLUSHINGS

INTRODUCTION

Various nutritional models in the gilt have shown that lower progesterone concentrations in early pregnancy are related to reduced embryonic survival (Ashworth et al., 1991; Pharazyn, 1992; Jindal et al., 1996; Almeida et al., 2000) and that exogenous progesterone therapy can restore embryonic survival (Ashworth et al., 1991; Jindal et al., 1997). Furthermore, there appears to be critical window of time in the immediate post-ovulatory period when nutritionally-mediated differences in progesterone concentrations affect embryonic survival (see Foxcroft, 1997), during which gamete transport, fertilization and early embryonic cleavage is occurring in the oviduct.

Accumulating evidence suggests that the oviduct is an active secretory organ that plays a role in fertilization and early embryonic development. As early as 1987, Gandolfi and Moor showed that in vitro co-culture of sheep embryos with oviductal cells improved the proportion of embryos developing to the blastocyst stage. Co-culture of oviduct epithelial cells and/or oviduct fluid with gametes or embryos in vitro has since been shown to be beneficial to fertilization (Nagai and Moor, 1990; Dubuc and Sirard, 1995; Way et al., 1997; Vatzias and Hagen, 1999), and embryonic development (Xu et al., 1992; Hwu et al., 1998). Progesterone and estrogen induce the synthesis and secretion of oviduct proteins, and possibly have a role in gamete maturation, fertilization, and embryonic development (see reviews by Nancarrow and Hill, 1995; Buhi et al., 1997). In Chapter 3, we showed temporal differences in IGF-I and Porcine Oviductal Secretory Proteins 1-3 (collectively termed POSP) mRNA expression, and in the secreted proteins, in oviduct flushings, with respect to time of ovulation in gilts. These differences were associated temporally with peripheral steroid concentrations and the E:P ratio.

POSP are members of a major family of proteins secreted by oviduct cells during the peri-estrus period (see review by Buhi et al., 1997). Counterparts to POSP have been characterized in cattle (Sendai et al., 1994), sheep (DeSouza and Murray, 1995), the mouse (Sendai et al., 1995), and in human (Arais et al., 1994) and exhibit a high degree of homology within conserved sequences to POSP (Buhi et al., 1996). In the cow (Boice et al., 1992), mouse (Kim et al., 1996) and pig (Buhi et al., 1993), these proteins have been shown to bind to zona pellucida, oocyte membrane, and to be associated with the perivitelline space in the pig (Buhi et al., 1993). In vitro studies with purified oviductal secretory proteins have demonstrated beneficial effects in several species on sperm-egg binding (Schmidt et al., 1997; Martus et al., 1998), and early embryonic development (Wallenhaupt et al., 1996).

IGF-I is also produced by oviduct cells in the pig (Wiseman et al., 1992) and cow (Winger et al, 1997) and is considered to be embryotrophic (see reviews by Schultz et al., 1993; Heyner, 1997). Like insulin, IGF-I has anabolic affects on cells, and its steroidogenic, mitogenic and trophic properties are well known (see review by Jones and Clemmons, 1995), although its role in the early developing embryo has yet to be fully elucidated. The biological action of IGF-I is modulated through high-affinity receptors and specific binding proteins that regulate its availability to certain tissues (Pfeifer and Chegini, 1994). The early developing embryo and oviduct express IGF Binding Proteins (IGFBP) and the IGF-I receptor in the cow (Winger et al., 1997), demonstrating the potential for maternal IGF-I to support pre-attachment development. IGF-I improved the development of human embryos to the blastocyst stage (Lighten et al., 1998), and bovine embryos to the morula stage (Matsui et al., 1995) in vitro, providing a role for IGF-I in early embryonic development.

We know that nutritional effects on embryonic survival can be mediated by steroid concentrations in the post-ovulatory period, and there are steroid-associated changes in POSP and IGF-I in the peri-ovulatory period (Chapter 3). In addition, the oviduct is exposed to higher concentrations of progesterone than the peripheral circulation (Hunter et al., 1983; Pharazyn et al., 1991; Chapter 2) because of the sub-ovarian countercurrent multiplier system (see review by Krzymowski et al., 1990). However, we lack evidence for effects of the local steroid environment on POSP gene expression, and on secreted POSP and IGF-I in oviduct flushings, which would confirm the functional importance of the sub-ovarian countercurrent system.

A serendipitous finding of Pharazyn et al. (1991) was that in vivo, plasma progesterone concentrations in the oviductal circulation ipsilateral to an ovary bearing no corpora lutea were not elevated compared to peripheral plasma concentrations. In addition, Wijayagunawardane et al. (1998) showed that in cows bearing only one corpus luteum, differential steroid concentrations existed in oviductal tissue ipsilateral and contralateral to the ovary with the corpus luteum, and that a relationship existed between the luteal and oviductal content of progesterone. Thus far, local regulation of the oviduct environment by ovarian steroids has been demonstrated in the cow (Binelli et al., 1999) and in the pig (Nichol et al, 1997). Therefore, we hypothesized that the oviductal environment could be affected by local ovarian steroids, suggesting that the oviduct could play a role in steroid-mediated embryonic loss.

To test this hypothesis, gilts were unilaterally ovariectomized to remove the local source of ovarian steroids from one oviduct. Then, during a narrow window of time after ovulation, oviduct flushings were collected to determine whether POSP and IGF-I and total protein concentrations in oviduct flushings were affected by local ovarian steroid concentrations, thus confirming the functional significance of the sub-ovarian countercurrent multiplier for oviduct function in the pig. These results, together with insight into temporal changes in IGF-I and POSP in oviduct flushings from Chapter 3, would allow us to determine whether nutritional effects in embryonic survival might be mediated by changes in the oviduct environment.

MATERIALS AND METHODS

Animals

Twenty-one cyclic Camborough x Canabrid terminal line gilts (Pig Improvement (Canada) Ltd.) used in this experiment were housed at the University of Alberta Swine Research Unit, in barns with a totally controlled environment and individually fed 2 X Energy Maintenance requirements of a standard grower diet. The experiment was run as two separate replicates (n=10 for replicate 1; n=11 for replicate 2). All animals were cared for in accordance with CCAC Guidelines with authorization from the Faculty Animal Policy and Welfare Committee. At around d 7 of their first estrous cycle, gilts were unilaterally ovariectomized under general anesthesia, taking care to remove all ovarian tissue whilst leaving the oviduct and oviductal vasculature intact. Gilts were then allowed to complete one full estrous cycle to allow for ovarian compensation. Estrus detection was carried out every 12 h starting at d 18 of the next cycle using backpressure testing during fenceline contact with a vasectomized boar. The onset of estrus was determined as the time of occurrence of a standing reflex in the presence of a boar, minus 6 h. Beginning at 12 h after the standing reflex was first observed, the remaining ovary in each gilt was examined by transcutaneous ultrasonography (Pie Medical Scanner 200, model 41480, Can Medical, Kingston, Ontario), using a 5.0-7.5 MHz multiple scan angle transducer for the presence of preovulatory follicles. Each gilt was scanned every 8 h until ovulation was completed. Time of ovulation was defined as the first scanning when no presumptive ovulatory follicles were seen, minus 4 h. Due to the number of follicles on the remaining ovary after compensation, it was common for some follicles not to ovulate.

Sample Collection

12 to 26 h after ovulation, heparinized blood samples from the jugular vein, and the oviduct veins ipsilateral (INT) and contralateral (OVX) to the remaining ovary, were taken under general anesthesia. In Replicate 1 only, additional samples were taken from a uterine vein and mixed arterial and venous blood from the ovarian

pedicle. Blood samples were centrifuged (2200xg, 4°C) and plasma separated and stored at –30°C until assayed for progesterone, estradiol and IGF-I concentrations. Both INT and OVX oviducts were flushed with 5 ml of sterile 0.15M NaCl (physiological saline) solution. The procedure involved inserting a blunted 18G needle attached to a 5 ml syringe into the lumen of the oviduct via the utero-tubal junction and occlusion to prevent fluid from entering the uterus. A flared collection funnel designed to provide an effective collection technique (courtesy of Dr. Vern Pursel) attached to silastic tubing (O.D. mm) was inserted approximately 0.5 cm into the ampulla, through the fimbria. The collection funnel was clamped by hand to prevent leaking during the flushing procedure and flushings were collected in 15-ml sterile graduated centrifuge tubes. Once the 5ml were injected into the oviduct lumen, the fluid was massaged towards the collection tubing to ensure most of the fluid was recovered. The fluid was immediately placed on ice and later stored at -30°C until further analysis. The gilts were then terminated with pentobarbitol (Euthansol) and oviducts were immediately dissected from the reproductive tract. Oviducts were trimmed free of connective tissue and midsections of the isthmus and ampulla were snap frozen in liquid nitrogen, and stored at -70°C.

Radioimmunoassays

Plasma progesterone concentrations were determined, in duplicate using an established radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corporation, U.S.A.), previously validated for use with porcine plasma without extraction (Mao and Foxcroft, 1998). Oviductal plasma samples were pre-diluted 10- and 50-fold with zero calibrator provided with the kit before being taken into the assay in duplicate. The sensitivity of the assay, defined as 89.9 % of total binding, was 0.094 ng/ml. The intra- and inter-assay CV were 10.04 % and 16.92 %, respectively.

Estradiol-17ß was extracted from 1 ml oviductal and peripheral plasma samples by the addition of 5 ml diethyl ether (VWR Canlab, Mississauga, ON, Canada) and vortexing for eight 1-min pulses. Oviductal fluid was pre-diluted 4-

and 8-fold before extraction with assay buffer (phosphate buffered saline containing 0.1% (w/v) gelatin, pH 7.0). The determination of estradiol-17ß was then performed by the radioimmunoassay procedure described in Chapter 3. Recovery of radiolabelled hormone was 83.7 ± 11.7 % efficient, and samples were not corrected for recovery. Treatments were balanced across assays to eliminate effect of assay on results. The assay sensitivity defined as 85.8 % of total binding, was 0.39 pg/ml and inter- and intra- assay coefficients of variance were 22.1% and 7.8 %, respectively. The estrogen:progesterone (E:P) ratio was calculated as the estradiol concentration / progesterone concentration in a particular sample.

IGF-I concentration in peripheral and oviduct plasma was determined using the homologous double antibody radioimmunoassay described previously in Chapter 3. The single assay used had an intra-assay coefficient of variance of 6.9% and the sensitivity defined as 93.7% bound, was 0.015 ng/tube. Recovery efficiency was 86.6 ± 2.5% and samples were not corrected for recovery.

IGF-I concentration in oviduct fluid was determined using the same assay described in Chapter 3, with modifications to the extraction procedure. To concentrate the samples sufficiently for detection, 0.5 ml or 1 ml of oviduct flushings were lyophilized, reconstituted in 0.4 ml assay buffer (phosphate buffered saline containing 0.1% (w/v) gelatin, pH 7.0), and extracted to remove bound proteins. Finally, 300 µl of neutralized acid extract was taken into the assay to ensure that the binding produced by experimental samples fell on the linear portion of the standard curve. Diluted oviduct fluid samples showed parallelism to the standard curve. Recovery efficiency was 98.6 ± 4.4%, and samples were not corrected for recovery. The assay sensitivity for the single assay run, defined as 98.8% binding, was 0.00195 ng/tube and intra-assay CV was 11.46 %.

Oviduct fluid total protein determination and Western blotting

Oviduct flushings were processed and dialyzed as per the procedure outlined in Chapter 3. The samples were then assayed for total protein determination using BCA assay (Sigma) and freeze-dried in 50ug aliquots. 5 µg of total protein from experimental samples and from a positive (pooled oviduct fluid collected at estrus) and negative (pooled oviduct fluid collected at D 28 of pregnancy), control were loaded onto 1.5mm thick, 4%-10% (w/v) gradient SDS-PAGE gels in duplicate. The electrophoresis procedure, transfer of proteins onto blots, and the Western blotting procedure using polyclonal antibody against POSP (a gift from Dr. Buhi, University of Florida) were as described in Chapter 3. As Buhi et al. (1996) proposed that POSP 1, 2 and 3 are derived from the same gene, POSP 1, 2, and 3, will be collectively referred to as POSP. POSP protein bands were quantified using densitometric techniques (Molecular Analyst v2.01, Bio-Rad Labs, Richmond, CA) and expressed as a proportion of the positive control sample density and corrected for total protein loading on the corresponding silver-stained gel. The control sample was run with each blot to allow for standardization of blots and comparison across gels. The average densitometric value of duplicate samples was used for statistical analysis.

POSP expression analysis

A subset of 7 animals, based on timing of ovulation to surgery interval and oviductal progesterone and estradiol concentrations, were used for mRNA analysis of POSP.

Total RNA was extracted from ampulla and isthmus sections of INT and OVX oviducts as previously described in Chapter 3. Preparation and hybridization of Northern blots with a 0.8 kb cDNA probe for POSP (obtained from Dr. W.C. Buhi, Univeristy of Florida, Gainsville, FL) to assess steady-state mRNA levels, was also carried out as previously described in Chapter 3. The message detected by the probe encodes for POSP 1, 2, and 3 (Buhi et al., 1996). All RNA data were normalized for loading and expressed as POSP/28S RNA in arbitrary densotimetric units.

Statistical analysis

Normal distribution of data was checked by the UNIVARIATE NORMAL procedure of SAS (1990), and plasma progesterone and estradiol concentrations, IGF-I in oviduct fluid and total protein concentrations, were log transformed to achieve normal distribution. Plasma progesterone, estradiol and IGF-I concentrations at each sampling site were analyzed using the GLM procedure of SAS: the model included Site of sampling, and Replicate as the independent variables, Time after ovulation as the covariate, and variance across animals as the error term. Multiple comparisons of means were analyzed using the method of Scheffe. The volume recovered, total protein concentration, IGF-I concentration, and POSP abundance and concentration, for INT and OVX oviducts, were analyzed by the GLM procedure of SAS (1990), using Replicate, Treatment and Replicate by Treatment interaction as the independent variables, time after ovulation as a covariate, and the variation across animals as the error term. POSP mRNA abundance was analyzed using Treatment, Section (ampulla or isthmus) and Treatment by Section as independent variables, with Time after ovulation as a covariate, and variation across animals was the error term. Because of unequal sample sizes, Least Squares Difference test was used to compare differences of means. This test was only performed if the statistical model and effects of treatment were both significant ($P<0.05$). There was no Replicate or Treatment by Replicate effect in any model. All correlations were analyzed using linear regression analysis (SAS 1990). The data are presented as LSM (± SE of LSM).

RESULTS

A total of six gilts were excluded from the analysis. One gilt did not cycle after ovariectomy and two did not ovulate. Three others did not fit the unilateral ovariectomy model with respect to established differences in plasma progesterone and estradiol in the oviduct circulation ipsilateral and contralateral to the remaining ovary, suggesting surgical damage to the countercurrent multiplier system.

Therefore, 15 gilts (n=7 for Replicate 1, and n=8 for Replicate 2) were included in analysis. Due to sample availability, IGF-I concentrations in plasma and oviduct flushings were only performed on samples (n=8 gilts) from Replicate 2. Consequently, IGF-I concentrations in peripheral and oviductal plasma were only analyzed in jugular, and INT and OVX oviductal veins, as uterine and ovarian samples were not taken in Replicate 2.

Reproductive characteristics

The average cycle length prior to sample collection was 20.4 ± 0.4 d, and was not different between replicates. The ovulation to surgery interval (20.7 ± 1.0 h), or onset of estrus to ovulation (34.1 ± 1.9 h), was not different ($P>0.05$) between replicates. The average number of corpora lutea in Replicate 1 gilts was 13.3 ± 1.3. Due to misappropriation of records, ovulation rate data for Replicate 2 are not available.

Plasma Progesterone, Estradiol and IGF-I concentrations

Because of the differences in time after ovulation between gilts (range 13 to 26 h), time after ovulation was used as a covariant to standardize least squares means for estradiol, progesterone and IGF-I concentrations across gilts for statistical purposes. Also, there were no significant interactions for site by time, replicate by time, or replicate by site.

Steroid concentrations differed ($P=0.0001$) across site of sampling, with concentrations of ovarian plasma 40-(estradiol) to 400-(progesterone) times higher, and INT oviduct 7-(estradiol) to 40-(progesterone) times higher than peripheral plasma, confirming the existence of the countercurrent multiplier. Estradiol and progesterone concentrations in OVX oviduct and uterine plasma were similar to peripheral estradiol and progesterone concentrations (Figure 4.1). As a consequence of the differences in estradiol (E) and progesterone (P) concentrations in plasma, the E:P ratio was different ($P=0.0001$) across site of sampling. The E:P ratios in plasma from jugular (1.04 ± 0.16), uterine (0.71 ± 0.23), and OVX oviductal (0.94 ± 0.16) veins were not different ($P>0.05$) from each other; however, the E:P ratio at these three sites was different ($P<0.05$) from the INT oviductal vein (0.17 ± 0.15) and mixed ovarian blood (0.13 ± 0.23). The E:P ratios in INT oviductal veins and mixed ovarian blood were not different ($P>0.05$) from each other.

There were no differences ($P=0.52$) in plasma IGF-I concentrations across jugular, INT oviduct and OVX oviduct veins (Figure 4.1). However, irrespective of site of sampling, time after ovulation was negatively correlated with plasma IGF-I concentrations ($r= -0.71$, $P=0.0004$), demonstrating that IGF-I concentrations in peripheral and oviductal plasma were decreasing over time (Figure 4.2). In addition, progesterone and estradiol concentrations were highly correlated within sample ($r = 0.629$, $P=0.0003$), but neither were correlated with IGF-I concentrations in that sample.

Volume recovered, total protein and IGF-I concentrations of oviduct flushings

Total volume of flushings recovered was not different between site and replicate, (3.33 ±0.18 and 3.53 ± 0.24 ml for INT and OVX oviducts, respectively). However, total protein concentration was lower ($P=0.026$) in INT oviduct compared to OVX oviduct (Figure 4.3A). Absolute IGF-I concentrations did not differ between INT and OVX oviducts in raw flushing measurements (Figure 4.3B). IGF-I concentrations in oviductal plasma and in oviduct flushings were not correlated (Figure 4.2), and unlike IGF-I concentrations in oviductal and peripheral plasma, IGF-I concentrations in oviduct flushings were not related to time after ovulation (Figure 4.2). However, IGF-I concentrations in oviduct flushings were positively correlated with total protein concentration in oviduct flushings ($r= 0.509$, $P= 0.05$).

POSP protein in oviduct flushings

The Western blotting technique resulted in immunoreactive protein bands migrating at 88 kDa and 115 kDa (Figure 4.4), which correspond to the 85kDA POSP 1, and the 100kDA POSP 2-3 that migrate as one 115kDa band on a 1D SDS-PAGE gel (Buhi et al. 1989). The densitometric values (in arbitrary units) of immunoreactive bands corresponding to POSP 1 and POSP 2-3 were combined for analysis, and collectively termed POSP. Specific binding of the primary and secondary antibody was confirmed by the lack of immunoreactive staining in the negative control pool (data shown previously in Chapter 3). POSP abundance as measured by densitometry was lower (P=0.0001) in OVX oviductal flushings as compared to INT oviductal flushings (Figure 4.3C), when expressed as per µg total protein. However, when converted to POSP protein concentrations (POSP abundance X total protein concentration), there was no difference between INT and OVX oviducts (Figure 4.3D). There was no relationship between POSP abundance or POSP concentration and time after ovulation

POSP protein abundance in oviductal flushings was positively correlated with progesterone and estradiol concentrations within oviduct (Figure 4.5). POSP protein abundance was negatively correlated (r=-0.65, P=0.0001) with total protein concentration in flushings, whereas POSP concentration was positively correlated (r=0.75, P=0.0001) with total protein concentration in oviduct flushings.

POSP mRNA analysis

Northern blot analysis of POSP revealed hybridizing bands at approximately 2.25 Kb (Figure 4.6A). There were no differences in POSP mRNA expression in INT or OVX oviducts, or when comparing ampulla and isthmus sections (Figure 4.7). POSP mRNA expression was high until 26 h after ovulation, by which time expression of POSP mRNA had almost disappeared (Figure 4.6B). Lastly, the expression of POSP mRNA and POSP protein abundance or POSP concentration, were not related, demonstrating that synthesis and secretion of POSP may not be coincident in this model.

DISCUSSION

The results from this study provide evidence that oviduct function is regulated by local ovarian steroids, and confirm the importance of the sub-ovarian countercurrent system for increasing ovarian steroid levels in the oviductal circulation. The progesterone and estradiol concentrations from different sampling sites (Figure 4.1) demonstrate that the sub-ovarian countercurrent system is effective in redirecting these steroids from the sub-ovarian vasculature into the ovarian and oviductal arteries. Pharazyn et al. (1991) and data in Chapter 2 showed that this countercurrent system does not appear to extend into the uterine artery, as the uterine venous plasma contained only peripheral concentrations of estradiol and progesterone. Furthermore, the OVX oviductal veins also contained peripheral concentrations of progesterone and estradiol in the present study, confirming the lack of a sub-ovarian countercurrent effect due to unilateral ovariectomy. These findings are again consistent with the earlier observations of Pharazyn et al. (1991), that in a gilt with no ovulations on one ovary, elevated levels of progesterone were not present in its ipsilateral oviduct. The concentration of progesterone and estradiol in the INT oviduct are also consistent with the data of Pharazyn et al. (1991) and the results presented in Chapter 2. Therefore, the function of the sub-ovarian countercurrent system allows for increased concentrations of progesterone and estradiol in the oviductal circulation, and the potential exists for local regulation of oviduct function by the ovary. More importantly, the oviduct may be affected by local ovarian steroid concentrations, as Eiler and Nalbandov (1977) demonstrated that concentrations of progesterone were increasing 16 h before ovulation in the utero-ovarian vein, and progesterone concentrations in the oviductal circulation were temporally different from those in the peripheral circulation (Chapter 2).

The effect of local ovarian steroid concentrations in the oviduct may be manifested in alterations of all physiological properties of the oviduct, including contractility, vascular perfusion, formation of oviduct fluid, and synthesis and secretion of proteins. Evidence for local regulation of oviduct fluid formation was demonstrated by Nichol et al. (1997) who showed that unilateral ovariectomy in pigs affects pH of the oviduct fluid. In the cow, Binelli et al. (1999) found that the pattern of oviduct protein secretion was different between oviducts ipsilateral to a persistent dominant follicle and oviducts ipsilateral to the corpus luteum. This may explain differences in fertility seen in cows with persistent dominant follicles (Savio et al., 1993), and emphasizes the importance of local ovarian steroid concentrations in the priming of the oviduct for reproductive events. Local ovarian steroids can also affect embryo development, as embryos co-cultured with bovine oviductal epithelial cells obtained from oviducts ipsilateral to progesterone- verses estrogen- dominated cystic follicles resulted in a lower proportion of blastocysts (Kamishita et al., 1999). In this study, we found that local ovarian steroids affect the protein concentration and POSP protein abundance in oviduct flushings, confirming the functional importance of the sub-ovarian countercurrent system.

The INT oviduct had lower total protein concentration than the OVX oviduct, and this is either due to a lack of fluid synthesis in the OVX oviduct causing a build-up of protein, or to an increase in fluid synthesis because of a higher E:P ratio in the OVX side. As the formation of oviduct fluid is steroid dependent (Bishop, 1956), differential steroid concentrations in each oviduct would be expected to affect oviductal fluid volume and total protein concentration. High estradiol concentrations have been associated with the largest fluid volume in the oviduct (Lippes et al. 1981; Wiseman et al., 1992), but due to the flushing method, our technique was not sensitive enough to establish differences in fluid volumes. Although Lippes et al. (1981) reported an inverse relationship between protein concentration and fluid volume in the human fallopian tube, protein production in our study was maximal during estrus and related to serum estradiol concentrations. Similarly, de novo protein synthesis and secretion by explanted porcine oviducts was highest for tissue recovered at estrus and was associated with elevated estradiol concentrations (Buhi et al., 1989). Lastly, results from Chapter 3 show that the highest total protein concentration was seen in oviduct flushings in the pre-ovulatory period and associated with a higher E:P ratio. Taken together, these data suggest that the OVX oviduct had a higher total protein concentration than the INT oviduct, likely due to both increased protein synthetic capability and fluid synthesis. Because ovarian steroids were lower in this oviduct, we propose that oviduct fluid synthesis may be regulated partly by the E:P ratio rather than the absolute concentrations of both estradiol and progesterone.

Although POSP protein concentrations (per ml of flushings) are not different between INT and OVX oviducts, this is most likely due to differential oviductal fluid dynamics. Our data clearly indicate that even peripheral levels of steroids in the OVX oviduct were sufficient to drive synthesis and secretion of POSP. The profiles of oviduct proteins in the OVX oviduct would be similar to those of ovariectomized animals receiving exogenous steroid therapy, because exogenous administration of steroids would not create high concentrations of steroids resulting from the countercurrent multiplier. Oviducts obtained from ovariectomized gilts receiving exogenous estrogen have been shown to synthesize and secrete POSP (Buhi et al., 1992), which is consistent with our OVX oviduct results. However, as POSP abundance (per unit of total protein) was lower in the OVX oviduct, active secretion of specific oviduct derived proteins appears to be responsive to the higher concentrations of ovarian steroids normally present in the oviductal circulation. Therefore, our data demonstrate unequivocally that the presence of high physiological concentrations of steroids, as seen in the INT oviduct in the unilaterally ovariectomized model used in this experiment, are important for providing a unique environment for gametes and the developing embryo.

A positive correlation between POSP protein abundance and progesterone concentrations may seem contradictory, as POSP are estrogen-induced (Buhi et al., 1992) and decrease in abundance when peripheral progesterone concentrations are rising after ovulation (Chapter 3). Similarly, although POSP mRNA synthesis is estrogen-induced (Buhi et al., 1996), it was interesting to find that POSP mRNA persisted until at least 26 h after ovulation when oviductal progesterone was very high (Chapter 3; this study). However, this may simply relate to the measurement of steady-state gene expression, with the mRNA transcript being stable over a long period but not being actively transcribed. The relationship between POSP protein abundance and progesterone concentration was not related to time after ovulation, meaning that the higher progesterone concentrations were not a result of the sample being taken later in the time course. We suggest that higher progesterone concentrations would have been associated with higher estradiol concentrations in the INT oviduct before ovulation. Consequently, before ovulation the abundance of both POSP mRNA and protein in the INT oviduct would also have been higher than the OVX oviduct.

However, as there was no relationship between the POSP mRNA and protein abundance, synthesis and secretion of POSP appears to be regulated separately. In support of this hypothesis, we detected POSP message in similar abundance in both the ampulla and isthmus sections of the pig oviduct. These are similar to results reported by Buhi et al. (1996), who also showed that POSP mRNA was detected in ampulla and isthmus during estrus. However, they reported that POSP message was significantly greater in the ampulla. Interestingly, in both studies by Buhi et al. (1990) and Buhi et al. (1992), in which de novo synthesis of POSP was restricted to the ampulla region in porcine oviductal explants, POSP was measured as secreted protein in explant culture medium, not as message in oviduct tissue. This suggests that although message is present, POSP protein per se may not be synthesized and secreted in the isthmus. Taken together, the data discussed above suggest that POSP synthesis and secretion are differentially regulated as a result of unilateral ovariectomy. Local ovarian steroid concentrations are, therefore, physiologically important for providing a unique environment for reproductive events occurring in the oviduct.

In this study, there were no differences between oviductal and peripheral plasma IGF-I concentrations, suggesting that the ovary is not a source of IGF-I, or that IGF-I is not transferred through the countercurrent system. No other study thus far has examined whether ovarian IGF-I is transferred through the countercurrent system into the oviduct vasculature. However, Jesionowska et al. (1990) reported that there were no differences between IGF-I concentrations in ovarian and peripheral venous blood. Available evidence, therefore, suggests that as the ovary is not a significant source of IGF-I, the sub-ovarian countercurrent system would be an important mechanism for determining IGF-I concentrations in the oviductal circulation.

The significant negative relationship between IGF-I concentrations in oviductal and peripheral plasma and time after ovulation may, in part, be dependent on cyclic changes in ovarian function. Although Wiseman et al. (1992) reported that peripheral IGF-I concentrations were 72.9 ng/ml during estrus, compared to 57.7 ng/ml at non-estrus, this difference was not significant and they were unable to conclude whether the observed variations were cyclic. Almeida (2000) also reported peripheral plasma IGF-I concentrations at 104 ng/ml at d 15 of the cycle, and between 130 to 150 ng/ml during the peri-estrous period, but reported no differences in IGF-I due to previous nutritional treatment during the peri-estrous period. Therefore, we cannot determine the cause of the observed relationship between time after ovulation and declining IGF-I concentrations in this study.

IGF-I may be embryotrophic as demonstrated with human and bovine in-vitro culture (Matsui et al., 1995; Lighten et al., 1998) and is present in the oviduct fluid of pigs (Wiseman et al., 1992; this study). Although oviductal cells synthesize IGF-I in vitro (Lee et al., 1992; Wiseman et al, 1992; Xia et al., 1996; Winger et al., 1997), a few studies have reported very low and undetectable levels of IGF-I synthesis from oviductal explants (Lee et al., 1992; Lai et al., 1996), arguing that the oviductal epithelium may not be the only source of IGF-I in flushings. However, in this study, we were unable to correlate IGF-I concentrations in oviduct fluid with those seen in plasma, which is consistent with the results of Wiseman et al (1992) and Chapter 3, and suggests that concentrations of IGF-I in oviduct flushings are predominantly related to local synthesis and release of IGF-I within the oviduct. The presence of differential total protein concentrations between INT and OVX oviducts, and a positive relationship between IGF-I concentration and total protein concentrations, provides evidence for altered oviduct fluid synthesis, which may be influencing IGF-I concentrations in oviduct flushings.

It still remains unclear whether IGF-I expression in the oviduct was affected by unilateral ovariectomy. Northern blot analysis of IGF-I mRNA was not sensitive enough to detect the levels of mRNA present in oviduct tissue in this study (data not presented). Therefore, it is necessary to utilize a more sensitive approach, such as RT-PCR, for detecting IGF-I mRNA expression, in order to address this question. Expression of IGF-I in the oviduct is under steroid hormone control, as estrogen induces IGF-I expression in the porcine uterus (Simmen et al., 1990), and the highest levels of IGF-I expression in the oviduct are seen in the immediate pre-ovulatory period (Stevenson and Wathes, 1996; Gabler et al., 1998). However, Schmidt et al. (1994) reported that IGF-I expression in the oviduct was highest immediately after ovulation in the cow (Schmidt et al., 1994), suggesting that species differences in IGF-I expression exist. The question of whether local ovarian steroids can affect IGF-I expression in the oviduct clearly requires further investigation.

Major effects of unilateral ovariectomy seen in this study were differences in local ovarian steroids in oviductal vasculature, total protein concentration of oviduct flushings, and the secretion of POSP. Local ovarian steroids clearly exert their influence on the ipsilateral oviduct, and high steroid concentrations created by the countercurrent multiplier appear to be necessary for creating a unique environment in the oviduct around the time of ovulation in pigs. Alterations in steroid synthesis by the ovary could, therefore, affect oviductal function, and possibly play a role in fertilization and early embryonic development. In turn, nutritionally-mediated effects on the ovarian production of steroids, or their metabolic clearance, could affect oviductal function in the critical peri-ovulatory period.

Figure 4.1. Least squares means (± SE of LSM) for plasma progesterone (P;ng/ml), estradiol (E;pg/ml), and IGF-I (ng/ml) concentrations in different sampling sites; Jugular (n=15 gilts), Uterine (n=7 gilts), oviductal veins contralateral (OVX oviduct) (n=15 gilts) and ipsilateral (INT oviduct) (n=15 gilts) to the remaining ovary, and Mixed ovarian blood (n=7 gilts). IGF-I was not measured in uterine and mixed ovarian samples, and only in Jugular, OVX and INT oviduct veins (n= 8 gilts for each site).

^{a,b,c} means within hormone with different superscripts differ by site of sampling (P<0.05).

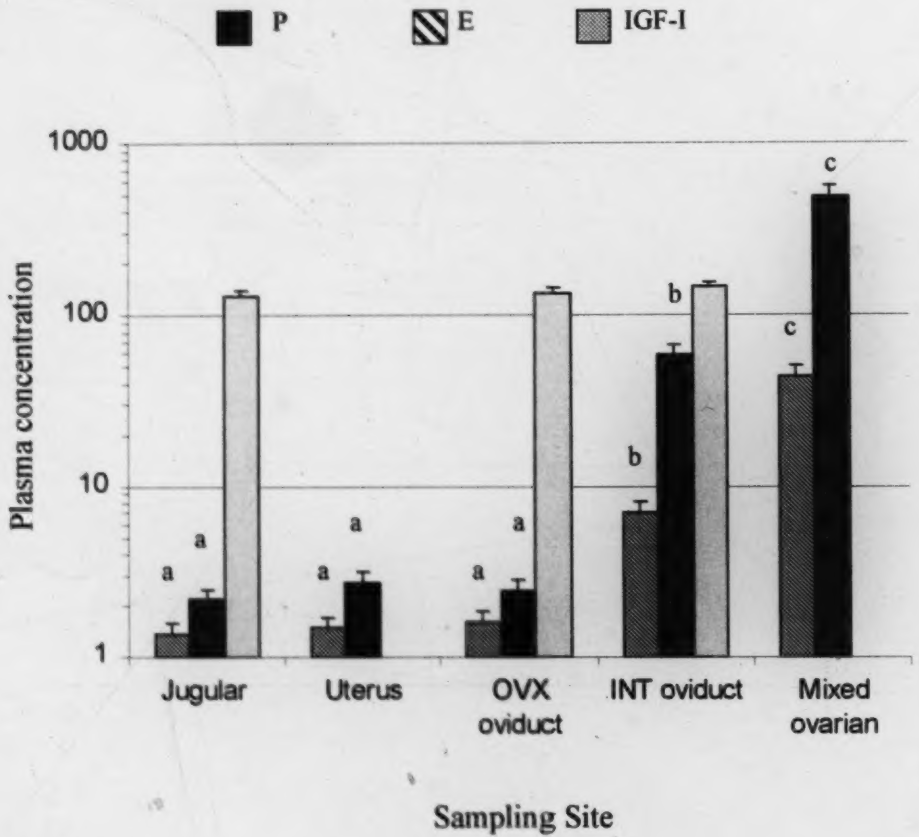


Figure 4.2. Relationships between IGF-I concentrations (ng/ml) in plasma and oviduct flushings and time after ovulation irrespective of site of sampling (n=8 gilts).

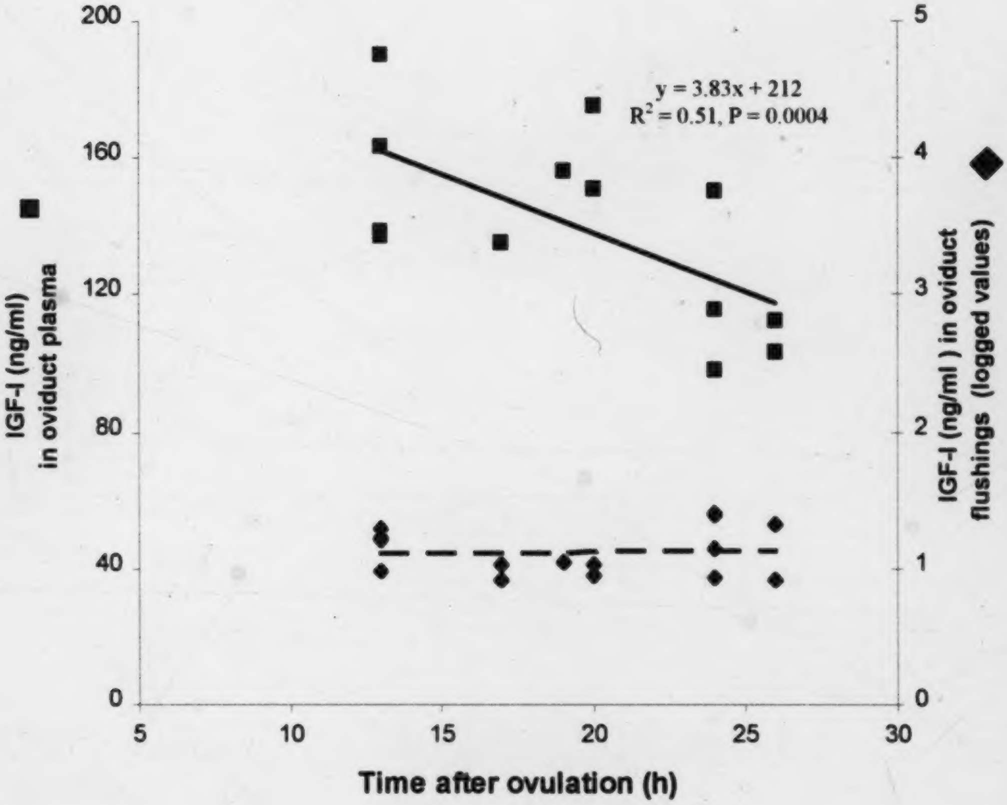


Figure 4.3. Comparison of **A)** Total protein concentration (n=15 gilts), **B)** IGF-I concentrations (n=8 gilts), **C)** POSP protein abundance (per µg total protein) (n=15 gilts), and **D)** POSP concentration (POSP abundance X total protein concentration) (n=15 gilts), in oviduct flushings between INT and OVX oviducts. Values are least squares means with error bars representing standard error of the mean.
* means differ ($P<0.05$)

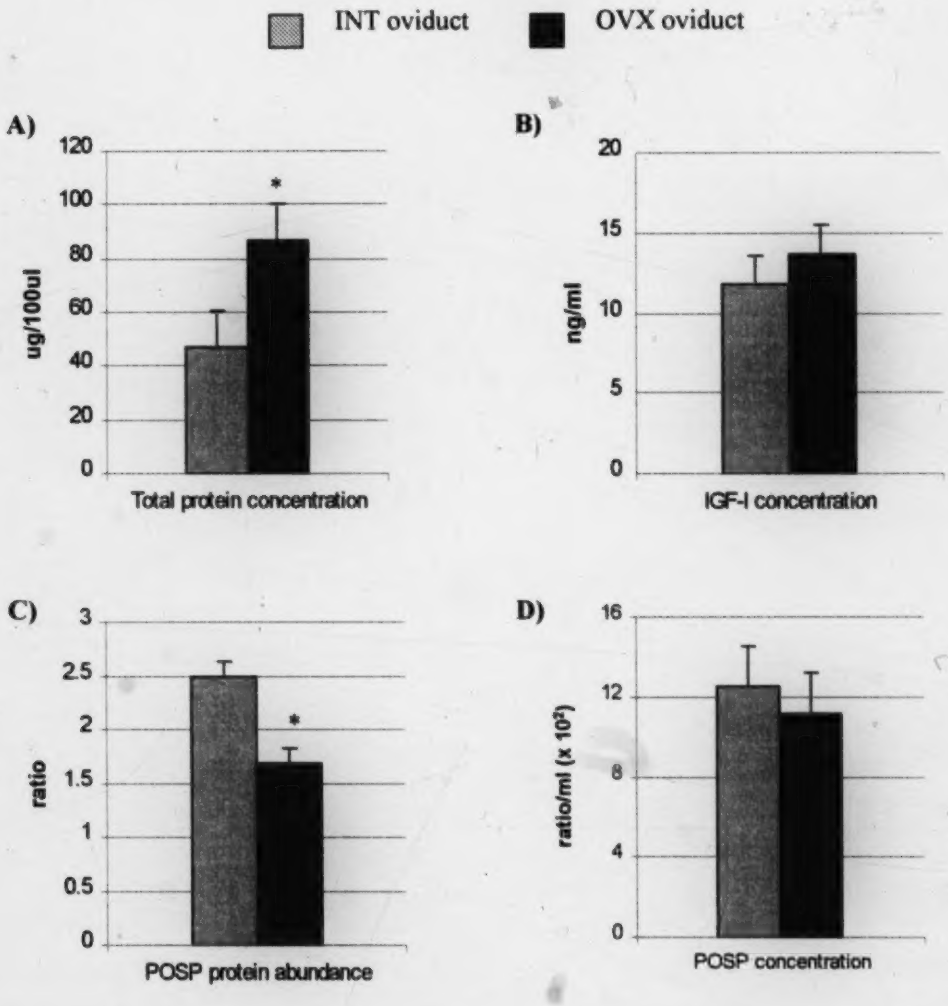


Figure 4.4. Representative Western blot of POSP proteins in oviduct flushings. Immunoreactive bands were detected migrating at approximately 88 kDa and 115 kDa. The 88 kDa band corresponds to POSP 1, and POSP 2-3 are the 115kDa band as they migrate together (collectively termed POSP) on 1-D SDS-PAGE gels. Oviduct flushings of five gilts are represented here, and within animal oviducts are labeled INT and OVX. Positive control (+) is indicated.

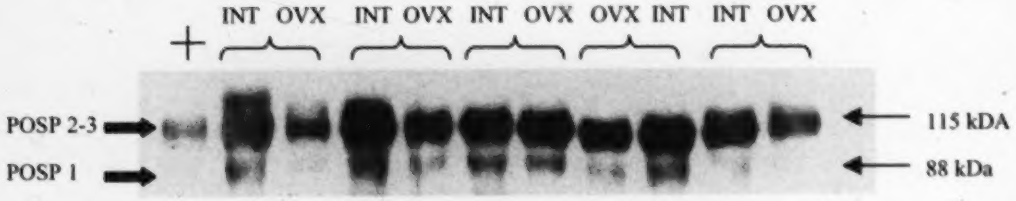
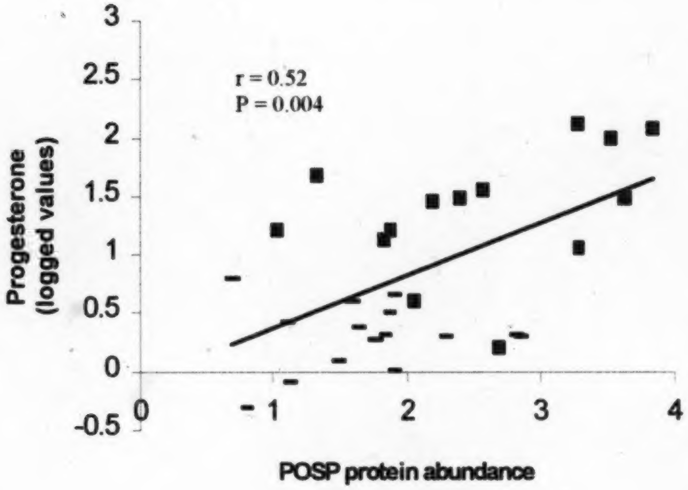

INT OVX INT OVX INT OVX OVX INT INT OVX
+
POSP 2-3
POSP 1
115 kDA
88 kDa

Figure 4.5. Correlation of oviduct vein progesterone concentrations (ng/ml) **(A)** and estradiol concentrations (pg/ml) **(B)** and POSP abundance (per µg total protein) within oviduct. OVX oviduct (–) and INT oviduct (■) are represented separately to show that the correlation is between, not within, oviducts.

A)



B)

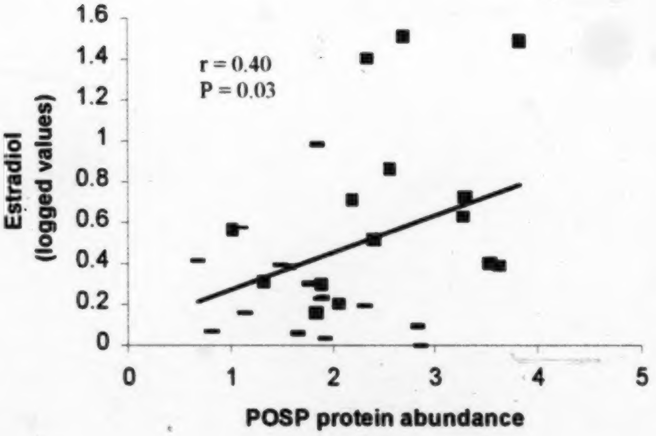


Figure 4.6. **A)** Northern blot of POSP mRNA expression. Samples on the gels were organized in relation to time after ovulation and site of origin; INT and OVX oviduct, and ampulla or isthmus. Each time point represents an individual animal, so treatment and section differences could be distinguished. **B)** There is no effect of time (P=0.20), but POSP mRNA expression appears to decrease 26 h after ovulation compared to all other time points.

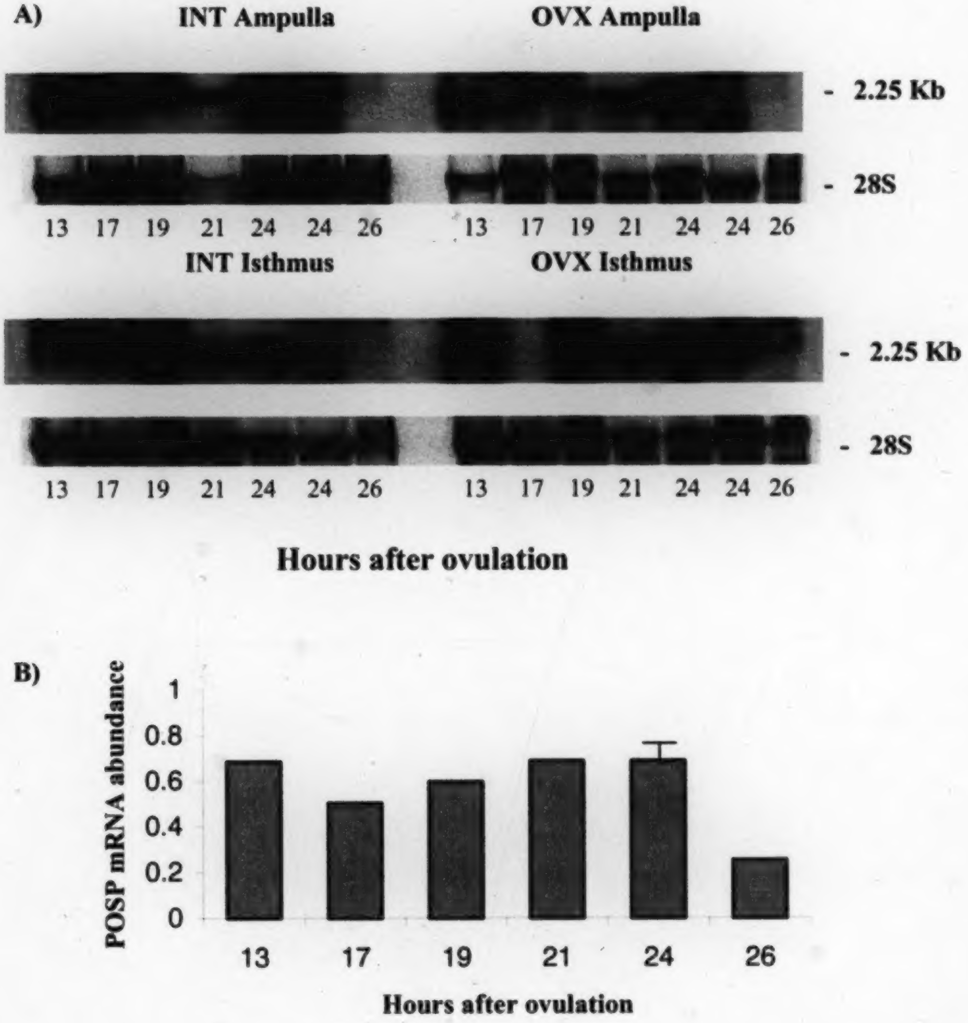
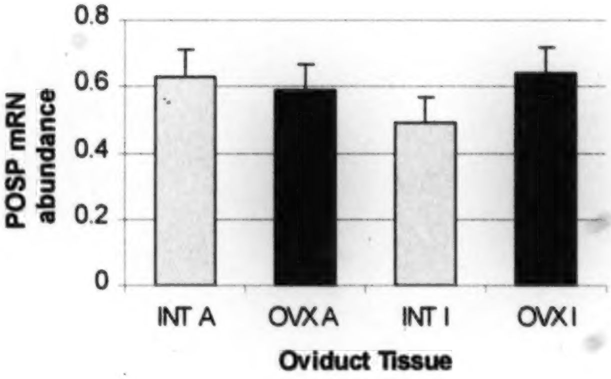


Figure 4.7. Least Square Means (± SEM) for POSP mRNA expression in INT ampulla (INT A), OVX ampulla (OVX A), INT isthmus (INT I) and OVX isthmus (OVX I); n=7 gilts per column.



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CHAPTER 5

EFFECTS OF PREVIOUS PATTERNS OF FEED INTAKE DURING THE ESTROUS CYCLE ON THE OVIDUCTAL ENVIRONMENT DURING THE PERI-OVULATORY PERIOD IN GILTS[1]

INTRODUCTION

Recent nutritional models have shown that different patterns of feeding of gilts in the cycle (Ashworth, 1991; Almeida et al., 2000) and after mating (Pharazyn, 1992, Jindal et al., 1996), can decrease subsequent embryonic survival at around day 28 of pregnancy, and embryonic loss in these studies was associated with lower progesterone concentrations in the first few days of pregnancy. Furthermore, exogenous progesterone therapy given in early pregnancy can counteract nutritionally induced embryonic loss (Ashworth, 1991; Jindal et al., 1997), demonstrating that differences in plasma progesterone may mediate nutritional effects on embryonic loss through steroid-dependent changes in the uterus and the oviduct (see review by Foxcroft, 1997). The oviduct may be particularly sensitive to changes in ovarian steroid secretion because of the existence of a sub-ovarian counter-current system in the pig, in which steroids are transferred from the ovarian vein draining to the ovarian and oviductal arteries (Krzymowski et al., 1982; Hunter et al., 1983). This system allows the oviduct to be exposed to high concentrations of steroids in advance of the peripheral rise of progesterone (Chapter 2).

The oviduct environment is not only affected by progesterone after ovulation, but also by estradiol during the peri-estrus period. Nutritional treatments imposed during folliculogenesis in the lactating and weaned sow (Zak et al., 1997; Yang et al., 2000) and cyclic gilt (Almeida et al., 2000) alter follicular development and steroidogenesis. Steroid-dependent changes in the oviduct may contribute to asynchrony of the embryo and the uterus (see reviews by Pope, 1988; Foxcroft, 1997), by changing the environment for embryonic development in the post-ovulatory period.

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[1] This chapter is part of a collaborative study where animal work and embryo developmental competence are reported in Almeida FRCL. Nutrition-reproduction interactions in cyclic gilts. Edmonton, Canada: University of Alberta; 2000. Thesis. A version of this chapter is to be published as a joint authorship with FRCL Almeida.

Co-culturing embryos in vitro with oviduct fluid improves the percent reaching the blastocyst stage in sheep (Gandolfi and Moor, 1987), reduces polyspermic fertilization in pigs (Nagai and Moor, 1990; Dubuc and Sirard, 1995), and improves implantation rates in humans (Yeung et al., 1996). Porcine oviduct secretory proteins 1-3 (POSP) are most abundant during the peri-estrus period (Buhi et al., 1989; Buhi et al., 1990), are estrogen-dependent (Buhi et al., 1992), and have been immunolocalized with the oocyte and remained associated with the embryo until the hatched blastocyst stage (Buhi et al., 1993). Counterparts to this protein have been characterized in other species, and are also thought to play a role in gamete viability, fertilization and early embryonic development (see reviews by Gandolfi, 1995; Nancarrow and Hill, 1995; Buhi et al., 1997).

IGF-I in oviduct fluid is also of interest, as its anabolic effects on various cell types are well-established (Jones and Clemmons, 1995). It is present in the oviduct fluid of pigs (Wiseman et al., 1992; Chapter 2), and appears to be derived from serum and oviduct synthesis (Wiseman et al., 1992; Xia et al., 1996; Winger et al., 1997). IGF-I increased the cleavage rate of embryos in vitro (Lighten et al., 1998), and possibly has a role in early embryonic development (see reviews by Schultz and Heyner, 1993; Heyner, 1997). It is unclear whether growth factors such as IGF-I function in an autocrine or paracrine manner to modulate oviduct function and embryonic development.

In a previous study by Almeida et al. (2000), littermate gilt trios, matched for growth rate, were either provided high feed intakes throughout the estrous cycle (HH), or feed-restricted in the first (RH) or second week (HR) of the cycle. The modest feed restriction during the second part of the cycle lowered embryonic survival at day 28 of pregnancy and was associated with lower progesterone concentrations 48 and 72 hours after onset of estrus. The objective of the present collaborative study was to gain insight into the mechanisms by which this pattern of feeding led to lowered embryonic survival, either through the quality of the oocyte (see Almeida, 2000) or changes in the oviduct environment (this Chapter).

MATERIALS AND METHODS

Animals

The experiment was conducted at the Swine Research Unit of the University of Alberta, in barns with a totally controlled environment, using 19 pairs of littermate gilts (Pig Improvement Canada Ltd., Camborough x Canabrid terminal line) in their second estrous cycle. The procedures for the selection, pre-treatment and nutritional management of gilts were as described by Almeida et al., (2000). However, the treatment comparison was limited to the RH and HR feeding regimens. Therefore, in the present study, littermate gilts were either allocated to feed restriction to 2.1 X energy requirements for maintenance from d 1 to 7 of the cycle, and then fed 2.8 X maintenance from d 8 until onset of estrus (RH); or fed 2.8 X maintenance from d 1 to 7, restricted to 2.1 X maintenance from d 8 to 15, and refed at 2.8 X maintenance from d 16 until onset of estrus (HR). All gilts were fed a wheat-barley-soybean grower diet, which was nutritionally balanced in terms of amino acids, vitamins and minerals to meet NRC (1988) recommended nutritional requirements (Composition of diets listed in Appendix 1).

Of the 38 littermate pairs initially allocated to this experiment, data from a subset of 20 littermate pairs was chosen for final analysis based on the criteria described by Almeida (2000). These criteria excluded gilts that did not show behavioural estrus after treatment (2 gilts), gilts with greater than 2-cell stage embryos at surgery (6 gilts), and sickness during treatment (1 gilt). The remaining 10 gilts were excluded from analysis in this study because of loss of oviduct fluid samples (4 gilts), and unmatched littermates (6 gilts). Therefore, only gilts that had littermates remaining in the study, and complete data sets were included. All experimental procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care and with authorization from the University Animal Policy and Welfare Committee.

Estrus and ovulation detection, and insemination

Starting at d 19 of the second estrous cycle, gilts were checked for estrus every 6 h (0600, 1200, 1800 and 2400) using the back pressure test during periods of fence line contact with mature vasectomized boars. Gilts were artificially inseminated 12 and 24 h after the first observed standing estrus with pooled semen from the same group of boars (Alberta Swine Genetics Corporation, Leduc, AB, Canada) specifically designated for this experiment. Time of ovulation was monitored using transcutaneous ultrasonography (Pie Medical Scanner 200, model 41480, Can Medical, Kingston, Ontario), using a 5.0 – 7.5 MHz multiple angle transducer to scan for the presence of pre-ovulatory follicles. Gilts were scanned every 6 h, beginning 24 h after onset of standing estrus, until completion of ovulation. Time of ovulation was defined as the first scanning when no presumptive ovulatory follicles were seen, minus 3 h.

Collection of oviductal plasma samples and oviduct flushings

12 to 20 hours after ovulation, gilts underwent surgery to recover early-fertilized oocytes for the study of early embryonic development reported by Almeida (2000), and oviduct flushings. Ovulation rates were recorded and each oviduct was flushed twice using 5 ml of Dulbecco's Phosphate Buffered Saline (DPBS, Sigma Chemical Company, St. Louis, MO, USA), previously warmed at 39° C. The procedure involved inserting a blunted 18G needle attached to a 5 ml syringe into the lumen of the oviduct via the utero-tubal junction and occlusion to prevent fluid from entering the uterus. A flared collection funnel designed to provide an effective collection technique (courtesy of Dr. Vern Persel) attached to silastic tubing was inserted approximately 0.5 cm into the ampulla, through the fimbria. The collection funnel was clamped by hand to prevent leaking during the flushing procedure and

flushings were collected in sterile Falcon dishes (Fisher Scientific, St. Louis, MO, USA). Flushing fluid was massaged towards the collection tubing to ensure that most of the fluid was recovered. A second 5-ml flush was then performed to ensure collection of all embryos but was not used for oviduct analysis. The oviduct flushings were then immediately transported to the laboratory in a Styrofoam box containing a tray and flasks filled with warm water to avoid cooling of the recovered oocytes. Embryos were removed and the remaining flushings were transferred into 15ml sterile Falcon (Fisher Scientific, St. Louis, MO, USA) centrifuge tubes, immediately frozen and were stored at –20°C until further analysis. Time from flushing of the oviduct to freezing of oviduct flushings was approximately 30 min.

Also at surgery, peripheral blood samples were collected by jugular venepuncture and oviductal blood samples were taken by venepuncture of a vein draining the mid-section of the oviductal vasculature. Heparinized blood samples were centrifuged (2200xg, 4°C), plasma separated and stored at –30°C until assayed for progesterone and estradiol concentrations.

Radioimmunoassays

Progesterone and estradiol-17ß concentrations were determined in duplicate from peripheral and oviductal plasma using the radioimmunoassay procedures described in Chapter 4. The sensitivity of the progesterone assay, defined as 97 % of total binding, was 0.098 ng/ml. The intra- and inter-assay CV were 9.3 % and 12.3 %, respectively. Recovery of radiolabelled hormone in the estradiol-17ß assay was 83.7 ± 11.7 %, and samples were not corrected for recovery. The estradiol assay sensitivity defined as 84 % of total binding, was 0.32 pg/ml and the intra-assay coefficient of variance for the single assay run was 7.2 %.

IGF-I concentration in peripheral and oviduct plasma was determined using the homologous double antibody radioimmunoassay described in detail in Chapter 4. The single assay used had an intra-assay coefficient of variance of 6.9% and the sensitivity defined as 93.7% bound, was as 0.015 ng/tube. Recovery efficiency was 86.6 ± 2.5% and samples were not corrected for recovery.

IGF-I concentration in oviduct flushings was also determined using the same assay (Chapter 4). Recovery efficiency was 98.6 ± 4.4%, and samples were not corrected for recovery. The assay sensitivity for the single assay run defined as 98.8% binding was 0.00195 ng/tube and intra-assay CV was 11.5 %.

Oviduct flushing protein determination and Western blotting

Oviduct flushings were prepared and assayed for total protein concentration based on the procedure outlined in Chapter 3. 5 µg of total protein from experimental samples and from a positive (pooled oviduct fluid collected at estrus) and negative (pooled oviduct fluid collected at d 28 of pregnancy) control, underwent electrophoresis under non-reducing conditions, proteins were transferred onto nitrocellulose membrane and Western blotted was applied according to the procedures outlined in Chapter 3. The blots were incubated with polyclonal antibody raised in rabbits against POSP 1-3 (a gift from Dr. Buhi, University of Florida). Protein bands for POSP 1-3 were quantified using densitometric techniques (Molecular Analyst v2.01, Bio-Rad Labs, Richmond, CA), the data grouped together and collectively termed POSP. POSP abundance (per µg protein) was expressed as a proportion of the positive control sample density and corrected for protein loading on the corresponding silver-stained gel. The control sample was run with each blot to allow for standardization of blots and comparison across gels. The average densitometric value of duplicate samples was used for statistical analysis. POSP abundance was also multiplied by total protein concentration in oviduct flushings from that oviduct to obtain a measure of POSP concentration (abundance/ml) in oviduct flushings.

Statistical analysis

Data were analysed as a randomized complete block design, each block consisting of two littermates representing each treatment, using the general linear model of Statistical Analysis System (SAS, 1990). Normality of data was tested by Shapiro-Wilk test (SAS, 1990), and data were log transformed as needed to produce a normal distribution for statistical analyses. Treatment and block were tested on time of surgery with respect to ovulation, ovulation rate, and volume recovered. Peripheral plasma measurements were tested using treatment and block as the independent variables, time after ovulation as a covariate, and variance across animals as an error term. For oviduct data, total protein concentration, IGF-I concentrations and POSP in oviduct flushings, and estradiol and progesterone concentrations in oviductal veins, were tested using treatment and block as the main effects, time after ovulation as a covariate, and variation across animals was used as the error term. Gilt was considered the experimental unit in all statistical analyses. Pearson correlation coefficients were used to establish relationships between measurements on individual oviducts, irrespective of gilt. In the event that significant treatment effects were established, multiple comparisons were performed using probability of differences (pdiff) between least square means, adjusted by Tukey-Kramer (SAS, 1990). All data are reported as Least Squares Means (± SEM of LSM).

RESULTS

Peripheral and oviductal plasma progesterone, estradiol and IGF-I concentrations

Peripheral and oviductal plasma progesterone, estradiol and IGF-I concentrations and the estradiol:progesterone (E:P) ratio are summarised in Table 5.1. There were no treatment differences, except for the E:P ratio in peripheral plasma, which was higher (P=0.04) in RH compared to the HR group. However, there was a significant difference in progesterone and estradiol concentrations between oviductal and peripheral plasma. There were no differences in IGF-I concentrations between oviductal and peripheral plasma, indicating lack of a multiplier effect for IGF-I. The effect of littermate was significant for IGF-I plasma concentrations (P=0.025) and the E:P ratio (P=0.003).

Oviduct flushings

There was no difference in total flushing volume collected from the oviduct (3.42 ± 0.14 and 3.36 ± 0.16 ml for HR and RH treatments, respectively).

IGF concentration in oviduct flushings was not affected by treatment, but showed a relationship with IGF-I in oviductal plasma (see Figure 5.1). There was also a significant effect of littermate on IGF-I in oviduct flushings (P=0.027). Total Protein Concentration in oviduct flushings was affected by treatment (P=0.002; Table 2).

The Western blotting technique resulted in immunoreactive protein bands migrating at 88 kDa and 115 kDa (Figure 5.2), which correspond to the 85kDA POSP 1, and the 100kDA POSP 2-3 that co-migrate as one 115kDa band on a 1D SDS-PAGE gel as described by Buhi et al., (1990). The immunoreactive bands corresponding to POSP 1 and 2-3, were corrected for protein loading, combined and collectively termed POSP, and all bands were expressed as a proportion of the positive control band on that blot. Specific binding of the primary and secondary antibody was confirmed by the lack of immunoreactive staining in the negative control pool as confirmed in Chapter 2 (data not shown). POSP abundance (per μg total protein) as measured by densiotometry was not affected by treatment (as shown in Table 5.2), but there was a significant (P=0.0001) littermate effect. POSP abundance was negatively correlated with progesterone concentrations in that oviduct, independent of gilt (Figure 5.3). POSP concentration [POSP abundance/μg total protein X total protein concentration (μg/ml); Table 5.2] was higher (P=0.008) in the RH group (295.8 ± 17.2) compared to the HR group (193.5 ± 17.19).

DISCUSSION

In this study, feed restriction during the second week of the cycle resulted in lower total protein and POSP concentrations in oviduct flushings, with no differences in POSP abundance, or IGF-I concentrations, or in peripheral and oviductal plasma progesterone, estrogen or IGF-I concentrations. This suggests that nutritional manipulation during the estrous cycle can change the oviduct environment after ovulation. However in this study, the effects are subtle, and apparently not associated with changes in steroid concentrations in the oviductal plasma at the time of surgery.

In our previous experiment, RH gilts had a higher embryonic survival rate and higher peripheral progesterone concentrations at 48, 72 and 96 h after onset of estrus than the HR group (Almeida et al., 2000a). However in the present study, peripheral progesterone concentrations were not different at any time after onset of estrus (Almeida, 2000), and there were no differences in peripheral progesterone concentrations at surgery, 12 to 20 h after ovulation. There is no reasonable explanation for the discrepancies in progesterone concentrations between the studies, as they were conducted with the same genotype, and in the same experimental conditions and facilities. Also, growth rate, feed intake, gilt weight and backfat changes were consistent between the two studies, as discussed by Almeida (2000). However, as embryonic survival was not determined in the present study, we do not know with certainty whether embryonic survival would have been different between HR and RH treatment groups, in the absence of differences in progesterone concentrations. Almeida (2000) found no differences between the two treatments in the development of recovered fertilized oocytes to the blastocyst stage in vitro, and concluded that either nutritional restriction in this study did not affect oocyte quality, as expressed in early developmental competence in vitro, or that differences in developmental competence of fertilized oocytes are not apparent up to the blastocyst stage of development in vitro. The first conclusion would be consistent with the lack of a treatment effect on plasma progesterone concentrations, assuming that progesterone mediates effects of previous nutrition on subsequent fertility. Unfortunately, this also precludes the possibility that steroid-mediated effects of treatment on the oviduct would be apparent, and it is understandable that there were only subtle changes in measured components of oviductal flushings. Other studies in ewes (McEvoy et al., 1995) and pigs (Graham et al., 1999) have been able to show nutritionally-induced differences in embryonic development in vitro up to the blastocyst stage, so it is possible that the nutritional treatment in this study was not severe enough to induce changes in follicular quality affecting subsequent embryonic development.

Total protein concentration was, however, lower in the HR group than the RH group, as was POSP concentration. However, there were no differences in POSP abundance between the two treatments and the changes in POSP concentration are largely due to the changes in total protein concentration. Oviduct synthetic ability is greatest during estrus (Buhi et al., 1989) and is associated with a higher E:P ratio (Chapter 2; Chapter 3). Maximal protein production in the oviduct is also reported to be coincident with highest fluid volume (Lippes et al., 1981), although our flushing technique was again not sensitive enough to determine differences in fluid volume. We suggest that the higher total protein concentrations in the RH group are due to an increased oviductal fluid and protein synthesis over the HR group at 12 to 20 h after ovulation. There are two possible explanations for the higher protein concentrations in the RH group. First, oviductal fluid synthesis could have been higher throughout estrus in the RH group, due to higher E:P ratios during follicular development reported by Almeida (2000). In Chapter 4, use of unilaterally ovariectomized gilts showed that oviducts with a higher E:P ratios in oviductal veins had higher total protein concentrations. Second, this could represent a shift in the profile of oviduct fluid synthesis in relation to ovulation. The temporal profile presented in Chapter 3, showed that total protein concentrations in oviduct flushings decrease sharply after ovulation, and were associated with the E:P ratio. Therefore, an alteration in the profile of estradiol, affecting the E:P ratio in the RH treatment group, would explain the differences in total protein concentration between treatment groups. Although the E:P ratio in oviductal plasma was not significantly different between treatment groups, the E:P ratio in peripheral plasma was higher in the RH group. In the present study, however, total protein concentration was not related to the E:P ratio. In either scenario this could be a result of the change in the peri-estrus profiles of estrogen established in a later collaborative study (Almeida, 2000), as a shorter interval between the estradiol peak and the LH peak, and the rise in progesterone concentrations, were associated with higher embryonic survival in sows (Soede et al., 1994). The HR group was nutritionally restricted during folliculogenesis and this could have altered follicular growth and hence steroidogenesis (Hunter and Wiesak, 1990). However, in the present study we did not measure steroid profiles before ovulation, so this cannot be substantiated.

In Chapter 4, we found that POSP abundance differed between oviducts ipsilateral and contralateral to the remaining ovary, and thus was regulated by estradiol and progesterone concentrations within that oviduct. As there were no differences in peripheral or oviductal estradiol and progesterone concentrations in the present study we would not expect differences in POSP abundance. However, there was a negative relationship between POSP abundance and oviduct plasma progesterone concentrations within each oviduct, which, as discussed earlier (Chapter 3) is consistent with POSP being estrogen-dependent. Also, this relationship was established irrespective of variance among gilts, suggesting that even in intact animals the oviductal environment is regulated independently by ipsilateral concentrations of ovarian steroids.

IGF-I concentrations in oviduct flushings were not different between RH and HR groups, which suggests that either treatment does not affect IGF-I concentrations, or the feed restriction was too modest to induce a difference in IGF-I concentrations in flushings. In previous studies, IGF-I concentrations were associated with total protein concentrations in oviduct flushings during the peri-ovulatory period (Chapter 3) and in unilaterally ovariectomized gilts 12 to 26 h after ovulation (Chapter 4). In this experiment, we have no reasonable explanation why there were differences in total protein concentrations but not in IGF-I concentrations in oviduct flushings. However, it is possible that the mechanisms regulating total protein concentrations and oviduct fluid synthesis are somewhat different than the mechanisms regulating IGF-I secretion into oviduct fluid. The E:P ratio may be regulating protein concentration and fluid synthesis as previously discussed, and IGF-I secretion, like POSP secretion, is regulated by absolute concentrations of estradiol and progesterone. In both previous studies (Chapter 3; Chapter 4), the differences in the E:P ratio, either temporally (Chapter 3) or in oviduct plasma (Chapter 4), were associated with differences in plasma progesterone and estradiol concentrations. In contrast, in the present study there were no differences in plasma progesterone or oestradiol; however the E:P ratio was different across treatments, possibly resulting in the lack of a relationship between IGF-I and protein concentrations in oviduct fluid. Nevertheless, it seems reasonable that the mechanisms regulating oviduct fluid synthesis would also affect IGF-I concentrations in oviduct fluid. Therefore, because the sampling time was narrow and we do not expect oviduct fluid dynamics to change substantially during this window of time, unlike in Chapter 3, it may have been difficult to observe a relationship between IGF-I concentrations and protein concentrations.

We did however, find a positive relationship between IGF-I concentrations in oviduct plasma and oviduct flushings. Previous studies have not shown associations between IGF-I in oviduct plasma and oviduct fluid during the estrous cycle (Wiseman et al, 1992) or oviduct flushings during the peri-ovulatory period (Chapter 3) in pigs. Both studies reported that plasma IGF-I levels did not vary throughout the estrous cycle and the peri-estrous period, however, IGF-I concentrations in oviduct flushings were highest during estrus (Wiseman et al., 1992). This time is also coincident with the highest oviduct fluid volume (Wiseman et al., 1992), and highest protein synthesis (Buhi et al., 1989), confirming the results of our previous study (Chapter 3), that oviduct fluid dynamics play a large role in the IGF-I concentration in oviduct flushings. Although no relationship has been observed between IGF-I in plasma and oviduct flushings over time, in the present study all samples were collected within 12-20 hours after ovulation and some association was established. This may be because oviduct fluid synthesis and secretion are not changing substantially over the collection period and, therefore, not affecting IGF-I concentrations. The association between IGF-I concentrations in oviduct plasma and flushings in this study suggest that some of the IGF-I found in oviduct flushings may originate from plasma. Although synthesis of IGF-I is highest from oviduct epithelial cells during estrus (Wiseman et al., 1992), we do not know how much this contributes to the IGF-I concentrations in oviduct flushings.

Lastly, our results confirm that estradiol and progesterone, and not IGF-I, are affected by the sub-ovarian countercurrent multiplier system. The countercurrent multiplier system functions to transfer ovarian steroids from the ovarian venous blood to ovarian and oviductal arteries (see review by Krzymowski et al., 1990), and concentrations in oviductal veins are 10-fold higher than in the peripheral circulation (Hunter et al., 1983; Pharazyn et al., 1991; Chapter 2, Chapter 4, this study). In the present study, IGF-I concentrations were again not different between oviductal and peripheral veins, confirming our results in Chapter 4 that the sub-ovarian multiplier does not function to raise IGF-I concentrations in the oviductal circulation. The significance of the counter-current multiplier system is in local regulation of oviduct function by ovarian steroids. Progesterone concentrations start to increase in advance of ovulation in the utero-ovarian vein (Eiler and Nalbandov, 1977), and after ovulation plasma progesterone is already high in the oviductal circulation in advance of rising progesterone concentrations in the peripheral circulation (Chapter 2, Chapter 4). This provides further evidence for a possible role of the oviduct in embryonic development, as ovarian steroidogenesis can affect the oviductal environment directly. This is further supported by the relationship between POSP abundance and progesterone concentrations (this study) and POSP abundance and progesterone and estradiol concentrations (Chapter 4) in an oviduct within animal.

In conclusion, nutritional restriction during the second week of the estrous cycle resulted in modest changes in oviduct flushings components, changing total protein concentrations but not POSP abundance, nor IGF-I concentrations. There were no differences in plasma progesterone, estradiol or IGF-I concentrations possibly due to the modest feed restriction imposed. However, POSP abundance was negatively associated with oviductal progesterone concentrations, demonstrating responsiveness of POSP secretion to declining plasma estradiol concentrations and increasing progesterone concentrations, and hence the E:P ratio, during this period. Also, IGF-I concentrations in oviduct plasma were correlated to IGF-I in oviduct flushings during this narrow window of time, suggesting that some of the IGF-I present in oviduct flushings is a serum transudate. The importance of the sub-ovarian countercurrent system was also demonstrated as local concentrations of steroids in the oviductal circulation can affect the ipsilateral oviductal environment. This may be a mechanism whereby steroid-mediated alterations in the oviduct environment can affect early embryonic development and hence embryonic survival.

Table 5.1. Least Square Means of peripheral and oviductal plasma progesterone, estradiol-17ß, and IGF-I concentrations at surgery. Pooled SEM for each measurement is indicated. No treatment[1] differences were observed ($P>0.05$).

^{a,b,x,y} Differences between sites of sampling within hormone are indicated with different superscripts ($P<0.05$)
*Differences within hormone, within site of sampling ($P<0.05$)

Parameter	RH	HR	Pooled SEM
Peripheral			
Progesterone (ng/ml)	1.97^a	2.43^a	0.21
Estradiol (pg/ml)	0.95^x	0.80^x	0.06
E:P ratio	1.17*	0.39*	0.45
IGF-I (ng/ml)	114.8	114.2	3.72
Oviductal			
Progesterone (ng/ml)	60.9^b	66.9^b	18.8
Estradiol (pg/ml)	8.13^y	9.14^y	2.96
E:P ratio	0.27	0.17	0.07
IGF-I (ng/ml)	115.8	111.6	4.40

[1] Treatments are defined as: RH: restrict fed to 2.1 X M from d 1 to d 7 of the estrous cycle, and then fed to 2.8 X M from d 7 until onset of estrus; HR: fed 2.8 X M from d 1 until d 7 of the estrous cycle, restrict fed at 2.1 X M from d 8 to d 15 of the estrous cycle, and then refed at 2.8 X M until onset of estrous

Table 5.2. Least Square Means of oviductal flushing total protein concentration, IGF-I concentration, and POSP abundance at surgery. Pooled SEM for each measurement is indicated beside treatment means.

^{a,b} means within row with different letter superscripts differ ($P<0.05$)

Parameter	RH	HR	Pooled SEM
Total Protein Concentration (ng/ml)	280.2^{a}	181.7^{b}	19.0
POSP abundance (ratio)	1.12	1.07	0.06
POSP concentration (abundance/ml)	295.8^{a}	193.5^{b}	17.2
IGF-I (ng/ml)	4.53	4.92	0.24

Figure 5.1. Relationship between IGF-I in oviduct flushings and oviductal plasma. The equation of the line is IGF-I concentration in oviduct flushings = 0.013(IGF-I concentration in oviductal plasma) + 3.21.

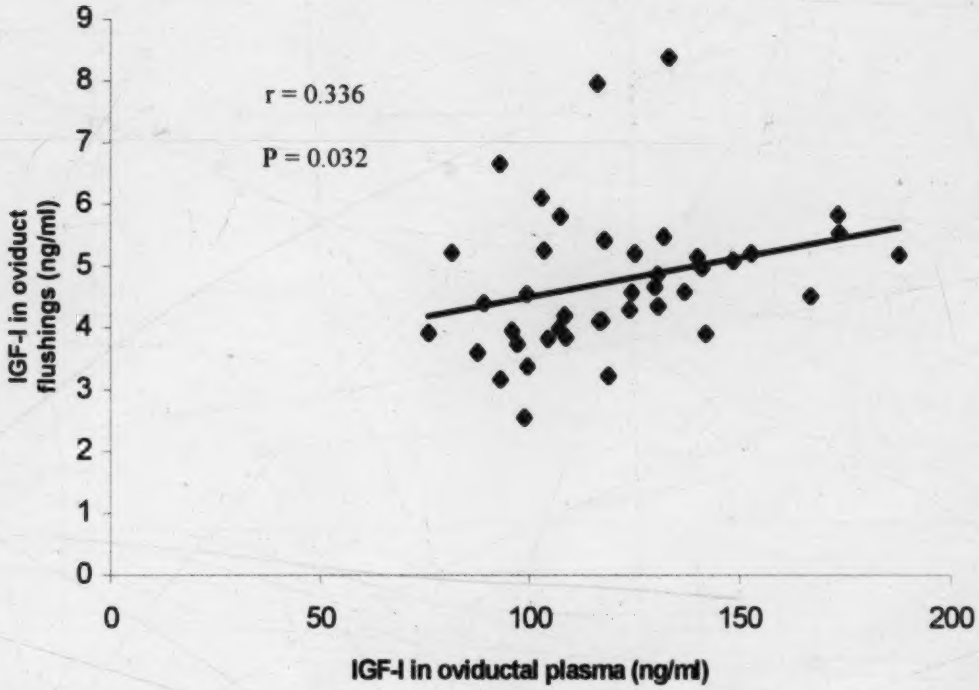


Figure 5.2. Representative Western blot of POSP abundance. Immunoreactive bands were detected migrating at approximately 88 kDa and 115 kDa. The 88 kDa band corresponds to POSP 1, and POSP 2-3 are the 115kDa band as they migrate together (collectively termed POSP) on 1-D SDS-PAGE gels. Oviduct flushings of eight gilts are represented here (four littermate pairs), and within animal oviducts are labeled right (R) and left (L). Positive control (+) is indicated.

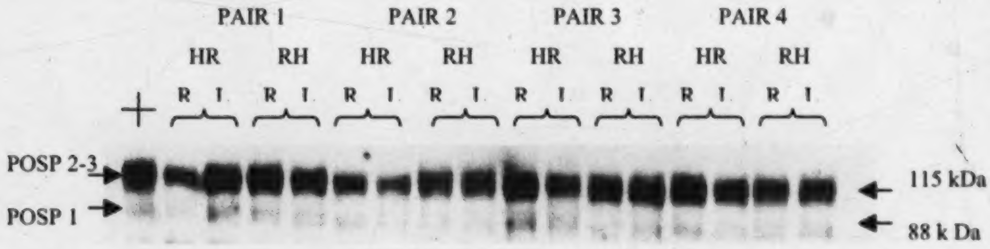
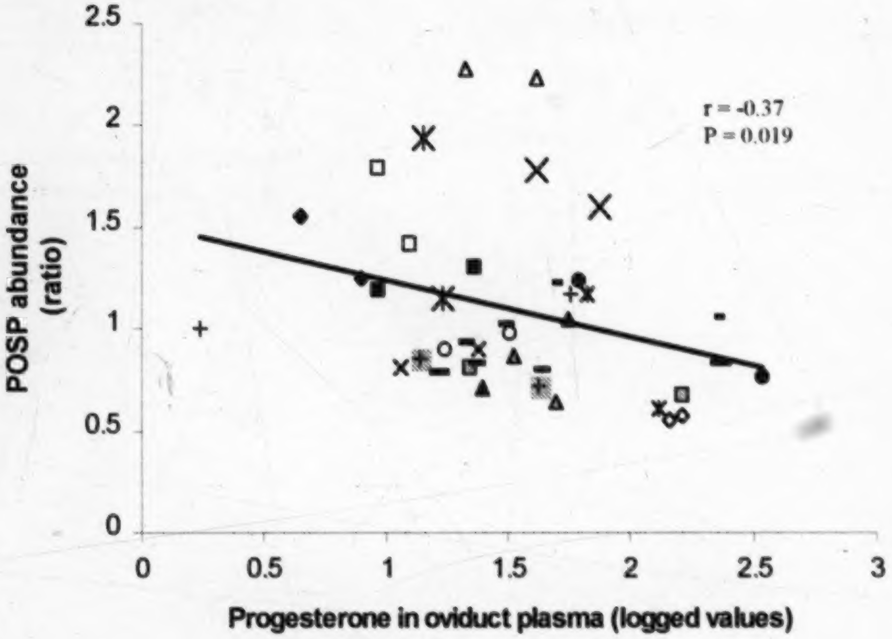

PAIR 1
PAIR 2
PAIR 3
PAIR 4
HR
RH
HR
RH
HR
RH
HR
RH
+
R L
R L
R L
R L
R L
R L
R L
R L
POSP 2-3
POSP 1
115 kDa
88 k Da

Figure 5.3. Relationship between progesterone in oviductal plasma and POSP abundance in oviduct flushings within each oviduct. The relationship (r = -0.371, P = 0.019) is negative, and independent of gilt. The two oviducts for each gilt are represented by the same symbol, demonstrating that the POSP abundance in each oviduct is regulated separately by the ipsilateral ovary.



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CHAPTER 6

FEED RESTRICTION AND INSULIN TREATMENT DIFFERENTIALLY AFFECT OVIDUCTAL PLASMA STEROIDS AND THE OVIDUCT ENVIRONMENT IN GILTS[1]

INTRODUCTION

Specific patterns of feed restriction during the estrous cycle (Almeida et al., 2000) and high feed intakes after ovulation (Ashworth et al., 1991; Pharazyn, 1992; Jindal et al., 1996) in gilts result in lowered embryonic survival at d 28 of pregnancy. Exogenous progesterone therapy in early pregnancy can in part restore embryonic survival (Ashworth et al., 1991; Jindal et al., 1997). A relationship between progesterone concentrations in the first few days of pregnancy and embryonic survival has been established in the gilt (Ashworth et al., 1991; Pharazyn et al., 1991a; Jindal et al., 1997) and in the sow (Van den Brande, 2000). Nutritional effects on embryonic survival in pigs have been extensively reviewed by Foxcroft (1997), and it is possible that altered nutrition during the estrous cycle affects folliculogenesis and oocyte quality, leading to reduced embryonic survival. Evidence for this was provided by Almeida (2000) as part of this collaborative study, as modest feed restriction during the second week, but not the first week of the estrous cycle, reduced the size of the pre-ovulatory surges of both estradiol and LH, and the rate of rise of plasma progesterone after ovulation. Decreased follicular growth may also affect luteal function, as postulated by Hunter and Wiesak (1990). Such effects were also established in a further extension of the present collaborative study in which Mao et al. (2000) demonstrated effects of both feed restriction and insulin treatment on luteal tissue cultured in vitro, and on the expression of genes regulating steroidogenesis.

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[1] The animals in this chapter are part of a larger, collaborative study, and data for the animal work is presented in Almeida FRCL. Nutrition-reproduction interactions in cyclic gilts. Edmonton, Canada: University of Alberta; 2000. Thesis.

In Chapter 3, we demonstrated that the oviductal environment changes during the peri-estrus period, and these changes are associated with the E:P ratio or plasma progesterone concentrations. Also, as discussed in Chapter 1, the sub-ovarian counter-current system in the pig (reviewed by Krzymowski et al., 1990) transfers ovarian steroids from the ovarian veins into the adjacent ovarian and oviductal arteries (Hunter et al., 1983; Krzymowski et al., 1982). We have also shown that progesterone concentrations are already high in the oviductal circulation, at a time when peripheral progesterone is rising (Chapter 2). Therefore, altered steroidogenesis and luteal function may affect oviductal function by changing the concentrations of steroids at the local oviductal level, and further evidence to support this concept has already been presented in Chapter 4 and Chapter 5.

The oviduct can play a role in embryonic development, as co-culturing embryos in vitro with oviduct fluid improves the percent reaching blastocyst stage (Gandolfi and Moor, 1987) and reduces polyspermy in oocytes when spermatozoa are co-cultured with oviductal cells (Dubuc and Sirard, 1995). Porcine oviduct secretory proteins 1-3 (POSP) are most abundant during the peri-estrous period (Buhi et al., 1989), are estrogen dependent (Buhi et al, 1990) and were immunolocalized in oocytes and remained associated until the hatched blastocyst stage (Buhi et al., 1993). Counterparts to these proteins have been characterised in other species, and are also thought to play a role in fertilization and early embryonic development (see reviews by Nancarrow and Hill, 1995; Buhi et al., 1997). We have shown that POSP protein abundance starts to decrease in oviductal flushings 48 h after ovulation (Chapter 3), and is related to oviductal progesterone concentrations (Chapter 4; Chapter 5). Also, the disappearance of POSP message in oviductal tissue precedes the disappearance of POSP protein by 24 h (Chapter 3; Chapter 4). Alterations in estradiol and progesterone concentrations in the oviductal circulation can, therefore, affect the timing and amount of POSP synthesis and secretion.

Insulin-like growth factor (IGF)-1 in oviduct fluid is also of interest as its mitogenic properties are well established. It is present in the oviduct fluid of pigs at estrus (Wiseman et al., 1992; Chapter 3; Chapter 4; Chapter 5), originating both from plasma and oviduct synthesis and secretion (Lee et al., 1992; Wiseman et al., 1992; Xia et al., 1996; Winger et al., 1997). The synthesis and secretion of IGF-I in the uterine epithelium is increased by exogenous estradiol and progesterone administration in the pig (Simmen et al., 1990), but this effect has yet to be established in oviductal tissue. Results from Chapters 3 and 4 showed that IGF-I concentration in oviduct flushings is strongly correlated to the peripheral E:P ratio, and it is related to oviduct fluid dynamics. The pathway for IGF-I action is reliant upon the presence of IGF-I receptors on the developing embryo, and IGF Binding Proteins (IGFBP) in the oviduct fluid may also modulate its action. IGF-I increases development of bovine (Matsui et al., 1995) and human (Lighten et al., 1998) embryos in vitro, and possibly has a role in early embryonic development. We were also interested in IGF Binding Protein (IGFBP)-4 expression in the oviduct, as it is expressed in the bovine oviduct (Winger et al., 1997) and is a inhibitor of IGF-I action (Jones and Clemmons, 1995).

The present collaborative study built on the previous study by Almeida et al., (2000), in which growth-rate matched, littermate trios were either fully fed throughout the estrous cycle (HH), or restricted in the first (RH) or second (HR) week of the cycle. The study showed that modest feed restriction during the second part of the cycle lowered embryonic survival at d 28 of pregnancy, and was associated with lower progesterone concentrations 48 and 72 h after onset of estrus. A further collaborative study by Almeida (2000), with only HR and RH treatment littermate pairs investigated how these patterns of feeding affected oocyte quality by measuring embryo developmental competence in vitro, and we also determined changes in the oviductal environment (Chapter 5). This study however, was not able to reproduce the lowered progesterone concentrations in the HR group that was previously reported, and similarly found no differences in embryo developmental competence. However, fertilization rate was modestly higher in the RH group, and the RH group had higher total protein concentration and POSP concentration in oviduct flushings than the HR group.

The present study was designed to repeat the same patterns of restricted feeding (HR and RH) in littermate trios, with the third littermate being fed as HR but receiving insulin injections during the period of feed restriction to counteract the potential negative effects of feed restriction on follicular growth and steroidogenesis. Insulin was used in this study because is able to restore follicular development in nutritionally-anestrous gilts (Britt et al., 1988), probably through its local actions on the ovary (Cosgrove et al., 1992; Whitley et al., 1998). Therefore, using material obtained as part of the overall study described in the paper of Almeida (2000), the objectives here were to determine if pattern of feed restriction in the estrous cycle and exogenous insulin treatment could affect the oviduct environment through total protein concentration, POSP synthesis and secretion, IGF-I concentrations and IGFBP-4 expression. Based on the extensive evidence presented in Chapters 2, 4, and 5, plasma progesterone, estradiol and IGF-I concentrations in the oviductal circulation were also determined and their associations with changes in the oviduct environment were investigated.

MATERIALS AND METHODS

Animals and Treatments

Almeida (2000) described the selection and management of gilts on experiment and treatment allocation in detail. All experimental procedures were carried out in accordance with the guidelines of the Canadian Council for Animal Care and with authorisation from the University Animal Policy and Welfare Committee. Briefly, 19 littermate trios of Camborough X Canabrid gilts (Pig Improvement (Canada) Ltd., Acme, AB, Canada) were randomly allocated to one of three treatments within litter. RH gilts were fed 2.1 X Maintenance Requirements from d 1 to d 7 of the estrous cycle, then feed was increased to 2.8 X Maintenance from d 8 to d 15. HR gilts were fed the opposite treatments, with 2.8 X Maintenance from d 1 to d 7, and 2.1 X Maintenance d 8 to d 15. HR+I gilts were treated as per HR treatment except were given long-acting insulin during the period of restriction. All gilts were returned to 2.8 X Maintenance from d 16 until breeding, at which point they were reduced to NRC (1988) guidelines for gestating gilts. Feed composition of diets is listed in Appendix 1.

Gilts were checked for onset of estrus every 6 h from d 18 of the treatment cycle, and were inseminated with pooled semen from the same three boars 12 and 24 h after the onset of estrus. Ultrasonography (Pie Medical Scanner 200, Can Medical, Kingston, ON) was performed every 6 h from 24 h after the onset of estrus to determine the time of ovulation.

Of the 57 gilts allocated to treatment, samples from 30 gilts (n=10 per treatment) were selected for analysis in the present study. The selected gilts were the same as those selected for the study of luteal function by Mao et al. (2000), on the basis of ovulation to surgery interval, peripheral plasma progesterone concentrations, and embryo developmental stage at surgery. Detailed endocrinological data during the treatment period and during estrus also exists for 23 of these 30 gilts (Almeida, 2000).

Collection of oviductal plasma samples and oviduct flushings

12 to 20 h after ovulation, gilts underwent surgery to recover early-fertilized oocytes and oviduct flushings. The surgical procedure included laparotomy and exposure of the uterine horns, oviducts and ovaries. The flushing procedure and embryo recovery has been described in detail in Chapter 5. Embryos were removed and cultured as described by Almeida (2000), and the first flushings were transferred into 15ml sterile Falcon (Fisher Scientific, St. Louis, MO, USA) centrifuge tubes, immediately frozen and were stored at –20°C until further analysis. Time from flushing of the oviduct to freezing of the oviductal flushings was approximately 30 min. Immediately after flushing both oviducts, ovariectomy was performed on the ovary with the largest number of ovulations. The ovary was removed and placed on ice for additional analysis of luteal function (Mao et al., 2000). Lastly, the associated oviduct was dissected free of connective tissue, and ampulla and isthmus samples were snap frozen in liquid nitrogen and stored at –80°C for later mRNA extraction and analysis as part of the present study.

Peripheral blood samples were also collected by jugular venipuncture and oviductal blood samples were taken by venipuncture of a vein draining the mid-section of the oviductal vasculature. Heparinized blood samples were centrifuged (2200xg, 4°C), plasma separated and stored at –30°C until assayed for progesterone and estradiol concentrations.

Radioimmunoassays

Plasma progesterone concentrations were determined, in duplicate, using an established radioimmunoassay (Coat-a-Count Progesterone, Diagnostic Products Corporation, U.S.A.), previously validated for use with porcine plasma without extraction (Mao and Foxcroft, 1998). Oviductal plasma samples were pre-diluted 10- and 50-fold with zero calibrator provided with the kit before being taken into the assay in duplicate. The sensitivity of the assay, defined as 91.4 % of total binding, was 0.09 ng/ml. The intra- and inter-assay CV were 10.0 % and 10.4 %, respectively.

Estradiol-17ß was extracted from 1 ml oviductal and peripheral plasma samples and assayed by the radioimmunoassay procedures described in Chapter 4. Recovery of radiolabelled hormone was 83.7 ± 11.7 %, and samples were not corrected for recovery. The assay sensitivity, defined as 84 % of total binding was 0.32 pg/ml, and the intra-assay coefficient of variance for the single assay run was 7.2 %.

IGF-I concentration in peripheral and oviduct plasma was determined using a homologous double antibody radioimmunoassay described previously (Chapter 4). The single assay used had an intra-assay coefficient of variance of 6.9% and the sensitivity defined as 93.7% bound, was as 0.015 ng/tube. Recovery efficiency was 86.6 ± 2.5% and samples were not corrected for recovery. IGF-I concentration in oviduct flushings was determined using the same assay with modifications to the extraction procedure as described previously (Chapter 3). Recovery efficiency was 72.7 ± 7.9%, and samples were corrected for recovery. The assay sensitivity for the single assay run, defined as 94.5 % of total binding, was 6 pg/ml and intra-assay CV was 10.5 %.

Oviduct fluid protein determination and Western blotting

Oviduct flushings were processed and total protein concentrations were determined as described in detail in Chapter 3. Experimental samples and positive (pooled oviduct fluid collected at estrus) and negative (pooled oviduct fluid collected at d 28 of pregnancy) controls underwent electrophoresis under non-reducing conditions, were blotted, and Western blot procedures were performed as described in detail in Chapter 3. The polyclonal antibody used was raised in rabbits against POSP 1-3 (a gift from Dr. Buhi, University of Florida), and this antibody has been reported to be specific for POSP 1-3 in pigs (Buhi et al. 1993). Protein bands corresponding to POSP 1, and 2-3 were quantified using densitometric techniques (Molecular Analyst v2.01, Bio-Rad Labs, Richmond, CA), combined for analysis and collectively termed POSP. POSP abundance (per unit total protein) was expressed as a proportion of the positive control sample density and corrected for protein loading on the corresponding silver-stained gel. The control sample was run with each blot to allow for standardization of blots and comparison across gels. The average densitometric value of duplicate samples was used for statistical analysis. POSP abundance was also expressed as a concentration (per ml flushings) by multiplying POSP abundance by total protein concentration of that sample.

POSP and IGFBP-4 mRNA analysis

Analysis of oviductal POSP and IGFBP-4 mRNA was successfully completed in oviduct tissues from 22 animals (8 HR, 7 HR+I, and 7 RH).

Total RNA was extracted from ampulla and isthmus sections of INT and OVX oviducts and Northern blotting carried out according to the procedures outlined in Chapter 3. Blots were hybridized using a 0.8 kb cDNA probe for POSP (obtained from Dr. W.C. Buhi, Univeristy of Florida, Gainsville, FL), or 0.5 kb cDNA probe for IGFBP-4 (obtained from Dr. Shunichi Shimasaki, University of California, San Diego, CA) to assess steady state mRNA levels. Blots were exposed to BioMAX MS film (Eastman Kodak, Rochester, NY) for 24 h for POSP and 1 week for IGFBP-4 at -80°C with 2 intensifying screens. Signal intensity was measured using densitometry techniques (Molecular Analyst v2.01, Bio-Rad Labs, Richmond, CA). All RNA data were normalized for loading and are expressed as signal intensity/28S band intensity.

Statistical analysis

Normality of data for all dependent variables was tested by the Shapiro-Wilk test (SAS, 1990), and data were log transformed as needed to produce a normal distribution before statistical analysis. Plasma data were analyzed as a complete randomized block design using the general linear model of Statistical Analysis System (SAS, 1990) and the complete model included littermate and treatment as the main effects, using ovulation to surgery time (time) as a covariate. Oviductal flushing data were analyzed using the same model, and variation across animals was the error term. In both cases, if time was significant ($P<0.05$) as a covariate, then time X treatment interaction was tested for significance ($P<0.05$), using time X treatment as a covariate. Lastly, with respect to oviductal tissue POSP and IGFBP-4 mRNA, treatment, littermate, and section (ampulla and isthmus) were tested as sources of variation, with time as a covariate, and variation across animals as the error term. To test time-dependent relationships on mRNA expression, treatment and time were tested as effects, and if time was significant in the model, then time by treatment interactions were tested to determine if slopes were heterogeneous between treatments. Gilt was considered the experimental unit in all statistical analyses. In the event that significant treatment effects were established, multiple comparisons were performed using probability of differences (pdiff) between least square means, adjusted by Tukey-Kramer (SAS, 1990). All correlations were performed using the linear regression analyses of SAS (1990), using normally distributed data. All data are reported as Least Squares Means (± SEM of LSM).

RESULTS

General

There were no treatment differences for time interval between ovulation and surgery (15.4 ± 0.7, 14.4 ± 0.7, and 14.9 ± 0.7 h for RH, HR and HR+I groups, respectively), ovulation rate (17.2 ± 0.8, 16.7 ± 0.8 and 18.2 ± 0.8 for RH, HR, and HR+I groups, respectively), or the time interval between onset of estrus and ovulation (41.2 ±1.6, 38.2 ±1.5, and 41.4 ± 1.5 h for RH, HR, and HR+I groups, respectively).

Peripheral and oviductal plasma progesterone, estradiol and IGF-I concentrations

Peripheral and oviductal plasma progesterone, estradiol and IGF-I concentrations and the E:P ratio are summarized on Table 6.1. Treatment effects on plasma hormones in Table 6.1 were limited to differences due to pattern of feeding (RH vs. HR), except that HR+I gilts had intermediate estradiol concentrations in oviductal plasma. IGF-I concentrations in both peripheral (P=0.01) and oviductal (P=0.03) plasma, and the E:P ratio in oviductal veins (P=0.05) were greater in RH gilts than HR and HR+I gilts.

There were no time by treatment interactions for any plasma components measured, but peripheral progesterone concentrations were related to time after ovulation (r= 0.34, P= 0.07). Littermate was significant for peripheral estradiol (P=0.0001), and IGF-I (P=0.017) concentrations, and the E:P ratio (P=0.0001), but not for peripheral progesterone concentrations. In oviduct plasma, effect of littermate was evident for IGF-I concentrations (P=0.0005) and the E:P ratio (P = 0.008), but not for estradiol or progesterone concentrations.

Oviduct flushings components

Least squares means for each treatment group for volume recovered, total protein and total protein concentration, and IGF-I concentration are presented in Table 6.2. There were no differences in volume recovered, but RH gilts had higher total protein (P=0.01) and protein concentration (P=0.0001) than HR and

HR+I gilts. Both total protein (P=0.0001) and protein concentration (P=0.0001) were affected by littermate.

IGF-I concentrations in oviduct flushings were affected by littermate (P=0.0007) and treatment (P=0.007), where RH gilts had the highest concentrations, HR gilts intermediate, and HR+I gilts had the lowest concentrations of IGF-I. Also, IGF-I concentrations in oviduct plasma and oviduct flushings were related (Figure 6.1).

The Western blotting technique resulted in immunoreactive protein bands migrating at 88 kDa and 115 kDa (Figure 6.2A), which correspond to the 85kDA POSP 1, and the 100kDA POSP 2-3 that migrate as one 115kDa band on a 1D SDS-PAGE gel as described by Buhi et al., (1990). The protein bands for POSP 1, 2, and 3 were quantified, combined and termed POSP. Specific binding of the primary and secondary antibody was confirmed by the lack of immunoreactive staining in the negative control pool (data shown in Chapter 3). POSP abundance was affected by littermate (P=0.0003) and by treatment (P=0.0009), as the insulin treated group had lower POSP/ µg total protein than the HR and RH groups, and HR was lower than RH (Table 6.2). Because of the effect of treatment on total protein concentration, with the RH group being higher than HR and HR+I groups, POSP concentration was highest (P=0.0001) in the RH group, lowest in the HR+I group and was intermediate in the HR group (Table 6.2). POSP abundance was also positively related (r=0.5297, P=0.0001) to IGF-I concentrations in oviduct flushings.

Oviduct tissue analysis

POSP mRNA expression was confirmed by the presence of a signal at approximately 2.25 kb, which corresponds to hybridization with the POSP cDNA probe (Buhi et al., 1996) (Figure 6.2B) and IGFBP-4 was confirmed by the presence of a single signal at approximately 505 bp (data not shown). A littermate effect was present for both POSP (P=0.01) and IGFBP-4 mRNA abundance (P=0.04). RH tissue had higher (P=0.006) POSP mRNA abundance than HR and HR+I tissue (Table 6.2) and there was also a time X treatment effect on POSP mRNA. The rate of POSP mRNA decrease over time in the RH group was faster (P=0.006) than in the HR and HR+I groups (Figure 6.3).

IGFBP-4 mRNA abundance was also affected by treatment (P=0.026) and was higher in the HR+I and RH groups compared to the HR group (Table 6.2). There was a possible time by treatment interaction with the rate of disappearance of IGFBP-4 mRNA being faster (P=0.08) for the RH group than the HR group, whereas IGFBP-4 mRNA was not related to time in the HR+I group (Figure 6.4).

DISCUSSION

Many studies have implicated changes in the oviduct environment as a mediator of the effects of nutrition and feeding on embryonic survival. Fasting of gilts for 48 h immediately after mating resulted in the proposal that embryos appeared in the uterus earlier than their fully-fed counterparts due to the affect of higher progesterone concentrations on oviduct contractions (Mburu et al., 1998). However, beneficial effects of higher progesterone concentrations in the peri-estrus period on embryo survival have been implicated in feed restriction after mating (Ashworth et al., 1991; Pharazyn et al., 1991a; Jindal et al., 1996). The effect of feed restriction in these latter studies would solely affect the events occurring after mating, suggesting the oviduct and uterine environments alone influence embryonic survival. Unfortunately, potential changes in the oviduct and uterine environment in these models have not yet been established, despite our efforts in Chapter 2 to replicate this experimental paradigm. In this study, the alternative model of feed restriction imposed during the estrous cycle was used, and therefore, may be affecting folliculogenesis and oocyte quality as well as the oviductal and uterine environments. This is one of the first studies to investigate the oviductal environment directly in vivo in response to previous nutritional treatment and its possible role in embryonic survival. In the present study, we have shown that feed restriction during the estrous cycle affected steroid profiles and the oviduct environment during the immediate post-ovulatory period. Also, insulin treatment differentially affected the oviduct environment compared to feed restriction, consistent with altered luteal function in insulin treated gilts (Mao et al., 2000). These changes in oviduct function provide evidence for a possible role of the oviduct in nutritionally induced embryonic survival.

In the experimental paradigm used for the present study, feed restriction in the second week, but not the first week of the estrus cycle resulted in lower progesterone concentrations and reduced embryonic survival (Almeida et al., 2000). In this case, the period of feed restriction that produces negative effects on subsequent fertility is coincident with emergence of follicles into the recruitable preovulatory pool (Hunter and Foxcroft, 1985). Nutrition affects follicular development, particularly steroidogenesis and subsequent luteal function, through metabolic hormones and metabolites acting centrally on hypothalamic regulation of LH (Booth et al., 1994), and through direct local effects on the ovary (see Cosgrove et al., 1992). As part of the present study (Almeida, 2000), we have already established that the RH and HR+I gilts had higher estradiol peak concentrations, a greater LH surge and a faster rise in progesterone after ovulation than HR gilts. Both greater follicle maturity before ovulation, and the greater LH surge, may result in faster lutenization and thus create a faster rise in progesterone in these animals. Also, in another part of this collaborative study, Mao et al. (2000) reported that luteal tissue obtained from RH and HR+I gilts responded to LH stimulation with increased release and production of progesterone in vitro. The results in Chapter 2 established the mechanisms by which lower pre-ovulatory estradiol concentrations, and a slower switch from estrogen to progesterone dominance as a consequence of reduced luteal function, may affect overall steroid priming of the oviduct, and thus affect oviductal environment.

This nutritional model used in this study was balanced for inherent littermate differences in reproductive capacity, which can be a source of large variations in reproductive parameters across animals. Thus, in this experiment, treatments were imposed on growth matched littermate trios, so the variation across litters was accounted for and treatment effects could be tested without this confounding effect. The importance of adopting this strategy is very evident from our data, as we were able to show a predominant littermate effect on all oviduct environment and tissue parameters measured, and on all peripheral and oviductal plasma components measured except peripheral progesterone and oviductal progesterone and estradiol concentrations.

In the present study, circulating ovarian steroids were not different at the same time point after ovulation between treatments in the peripheral circulation, which was consistent with analysis of a larger group of animals in this experiment (Almeida, 2000). However, using an analysis of time-dependent changes in progesterone, Almeida (2000) showed that the RH and the HR+I groups had a faster rise in peripheral progesterone concentrations after ovulation than the HR group. In addition, Almeida et al. (2000), using the same experimental paradigm showed that progesterone concentrations were lower in the HR group compared to the RH group 48 and 72 h after the onset of estrus. Nonetheless, higher peak estradiol concentrations in RH and HR+I gilts, compared to HR littermates in the present study, is consistent with higher oviductal E:P ratios in the RH group compared to the HR group, with intermediate levels in the HR+I group. Higher estradiol concentrations, as opposed to lower progesterone concentrations, also contributed to the higher E:P ratio in RH gilts, and this may contribute to alterations in oviduct function in these animals.

The oviductal concentrations of progesterone and estradiol were, as expected, higher than concentrations of those steroids in the peripheral plasma. The oviduct is exposed to higher levels of ovarian steroids than the peripheral circulation (Hunter et al., 1983; Pharazyn et al., 1991a; Chapter 2) and this is due to the presence of the sub-ovarian counter current system (Krzymowski et al., 1990). Lack of an ovarian source of steroids either through unilateral ovariectomy (Chapter 4), or an ovary bearing no corpora lutea in pigs (Pharazyn et al., 1991a), allows only peripheral levels of steroids to perfuse the oviduct. Unilateral ovariectomy results in differential pH in oviduct fluid (Nichol et al., 1997), and differential oviduct protein secretion and oviduct fluid properties (Chapter 4) in the pig, and lack of sperm transport into the oviduct ipsilateral to the ovariectomy in the rat (Sultan and Bedford, 1996). This evidence confirms the functional importance of the sub-ovarian counter current system on oviduct function, as postulated by Hunter et al. (1983). It also shows, in the context of this experiment, how the ovarian steroids produced during follicular development and after ovulation may directly affect the oviduct environment.

The late period of feed restriction (HR) was associated with lowered total protein and protein concentration in the oviductal flushings. We have previously shown that both total protein and protein concentrations were higher in the 48 hours prior to ovulation in oviduct flushings of gilts, coincident with higher E:P ratios in peripheral plasma of those gilts (Chapter 3). Similarly, our results from Chapter 5 also show that the HR group had lower protein concentrations, and a lower E:P ratio 12 to 20 h after ovulation compared to the RH group. This implies that the higher levels of total protein seen in the RH group are due to the greater estradiol peak concentrations and E:P ratios. Highest oviduct fluid and protein synthesis occur during estrus (Lippes et al., 1981; Buhi et al., 1989; Wiseman et al., 1992), which indicates that the RH gilts may have a more optimal oviductal environment during fertilization and early embryonic development. In Chapter 4, we showed higher protein concentrations in the flushings obtained from the oviduct contralateral to the remaining ovary, which were associated with the higher E:P ratio in that oviductal plasma POSP abundance was lower in oviductal flushings with the higher total protein concentration in Chapter 4; however, in this experiment, POSP abundance was highest in the RH group coincident with a higher total protein concentration. As discussed in Chapter 4, lowered POSP abundance in the ovariectomy model may be due to the absence of physiological concentrations of estradiol and progesterone in the oviduct ipsilateral to the ovariectomy.

In the present experiment, POSP protein abundance was also highest in the RH group and, intermediate in the HR group, but when expressed as a concentration, the POSP protein concentration was almost doubled in RH gilts compared to HR gilts. The POSP mRNA abundance was similar to POSP protein concentration, as steady state levels of mRNA were also doubled in the RH group compared to the HR group. POSP expression and secretion is estrogen-induced (Buhi et al., 1992; Buhi et al., 1996), and the RH group had higher oviductal estradiol concentrations at the time of sampling as a possible reflection of higher preovulatory peak estradiol concentrations (Almeida, 2000). This may be the reason for the higher POSP mRNA expression, protein abundance, and concentrations in oviductal flushings and tissue in the RH group.

Although the insulin treated gilts had higher pre-ovulatory peak estradiol concentrations similar to the RH gilts, insulin treatment was not associated with higher POSP mRNA expression or secretion, and HR+I gilts had the lowest POSP abundance. Also, there was a significant time by treatment interaction (Figure 6.2), whereby RH mRNA levels were strongly correlated with time and decreased rapidly compared to HR and HR+I gilts. Although this could suggest that insulin treatment either did not counteract the effects of feed restriction on POSP or was detrimental to oviduct function, insulin may be differentially affecting the oviduct environment compared to feed restriction. Evidence from a separate part of this study on luteal function shows that insulin treatment increased mRNA expression of key steroidogenic enzymes in the early luteal tissue recovered from the same gilts, and increased sensitivity to LH (Mao et al., 2000), which provides evidence for improved luteal function in insulin treated, as opposed to feed restricted gilts. mRNA for key enzymes responsible for progesterone synthesis were also already high in the HR+I group, while levels were increasing over time in the HR and RH gilts. In the present study, POSP mRNA was already low and decreasing slowly, and IGFBP-4 mRNA remained high and was not responsive over time, in the HR+I treatment. So, although oviduct fluid properties in the HR group are similar to the HR+I group, major differences in POSP protein abundance and POSP and IGFBP-4 mRNA levels, may indicate that insulin treatment advanced the oviductal environment with respect to time after ovulation, compared to the feed restricted groups.

The ability of previous pattern of feeding and exogenous insulin to affect the POSP mRNA expression and protein abundance, suggests that altered profiles of steroids during the estrus period and progesterone production after ovulation may adversely affect the oviductal environment and contribute to nutritionally induced embryonic loss. POSP becomes associated with the fertilized oocyte and developing embryo (Buhi et al., 1993), and Wollenhaupt et al. (1996) showed that a 97 kDa glycoprotein purified from pig oviductal fluid increased protein synthesis by 4-cell porcine embryos in vitro. Counterparts to this protein in other species have improved sperm-egg binding (O'Day-Bowman et al., 1996; Schmidt et al., 1997) and fertilization (Martus et al., 1998) in vitro, and possibly play a role in early embryonic development (see review by Buhi et al., 1997). Since POSP mRNA expression and secretion was higher in the RH group compared to other treatments during the same period of time after ovulation, this suggests that a better oviductal environment for developing embryos existed in the RH group. However, insulin seems to have altered POSP synthesis and secretion temporally due to increased luteal function, and this may also produce a beneficial effect on embryonic development.

As repeated in Chapters 3, 4, and 5, IGF-I concentrations did not appear to be affected by the countercurrent system, as similar levels were found in both the peripheral and oviductal plasma. This is consistent with the inability of unilateral ovariectomy to change IGF-I concentrations in the oviductal circulation (Chapter 4) in gilts and the study of Jesionowska et al. (1990), which showed no significant differences between IGF-I concentrations in peripheral and ovarian drainage in sows. Surprisingly, insulin treatment did not influence IGF-I concentrations in peripheral plasma, oviductal plasma or oviduct flushings. IGF-I concentrations in both peripheral and oviductal plasma were similar, and the RH gilts had higher IGF-I concentrations in both. Lower plasma IGF-I concentrations have been associated with feed restriction in pigs (Booth et al., 1994; Zak et al., 1997), but in this study IGF-I concentrations during the period of feed restriction and re-feeding at d 15 and d 16 of the treatment cycle were unaffected by treatment (Almeida, 2000). One explanation for the differences in plasma IGF-I concentration observed 12 to 24 h after ovulation could be that nutritionally-induced changes in folliculogenesis, although not evident in the period of follicular recruitment, could be further augmented during the later stages of preovulatory follicular growth. Therefore, the same mechanisms acting to lower IGF-I concentrations in the HR group, might be responsible for the differences in oviduct fluid properties reported in this experiment. However, it is unlikely that differences in ovarian IGF-I could contribute to the differences found in this study, as Wiseman et al. (1992) found serum IGF-I concentrations were not significantly linked to the estrous cycle although they ranged from 57.2 to 72.9 ng/ml during the cycle. Although we cannot provide an explanation for why IGF-I concentrations were affected by nutritional treatment after the period of feed restriction, but were not evident during restriction and re-feeding (Almeida, 2000), the source of these differences can be attributed to the consequences of feed restriction during the second week of the estrous cycle.

Irrespective of gilt or treatment, IGF-I concentrations in oviduct plasma and oviduct flushings were related (Figure 6.1). This is consistent with the results in Chapter 5, as a significant relationship between IGF-I in oviduct plasma and flushings was also observed. This indicates that some of the IGF-I present in oviduct flushings may be serum derived. Oviduct cells in vitro produce IGF-I in the pig (Lee et al., 1992; Wiseman et al., 1992) and in the cow (Xia et al., 1996; Winger et al., 1997), but it is not known how much the oviductal synthesis of IGF-I contributes to the IGF-I concentrations in oviduct fluid. It is also possible that the same mechanisms affecting IGF-I concentrations in plasma could be affecting IGF-I concentrations in oviduct flushings; however, Wiseman et al. (1992) did not find evidence of cyclic changes in IGF-I concentrations in plasma or in oviduct fluid. A higher IGF-I content during estrus was reported by Wiseman et al. (1992), however, this value was obtained by multiplying IGF-I concentrations by fluid volume, which was significantly higher at estrus. These findings further substantiate the nutritional effects on IGF-I concentrations in oviduct flushings demonstrated in this study, suggesting that oviductal IGF-I synthesis was lower in the HR group, or the HR treatment lowered overall IGF-I plasma concentrations and consequently IGF-I concentrations in oviductal flushings.

Also, insulin treatment increased IGFBP-4 mRNA levels to those seen in the RH group. So although IGFBP-4 mRNA mean levels were similar in RH and HR+I gilts, in the RH group IGFBP-4 mRNA decreased more rapidly over time compared to HR and HR+I gilts. IGFBP-4, like other IGFBPs, functions to transport IGFs appropriate sites of action, influences IGFs half-life, and modulates the affect of IGF action with receptors (Jones and Clemmons, 1995). IGFBP-4 is only thought to play an inhibitory role by suppressing action of IGF-

I, and it is associated with follicular atresia in the ovary (Grimes et al., 1994; de la Sota et al, 1996). Jones and Clemmons (1995) suggest that IGFBP-4 may beneficially serve to protect cells from overstimulation of IGFs, or to activate other pathways that are inhibited by IGF exposure. Higher IGFBP-4 mRNA expression without higher levels of IGF-I in the HR+I group do not support this hypothesis, but not enough is known about the function of the IGF system in the oviduct to determine whether higher IGFBP-4 mRNA expression is detrimental or beneficial to embryo development. IGF-I was shown to increase morula development of bovine embryos (Matsui et al., 1995) and blastocyst development of human embryos (Lighten et al., 1998) in vitro. For IGF-I to affect porcine embryo development, the embryo must possess receptors for IGF-I. However, the presence of IGF-I receptors at this stage of embryo development is not known in the pig. Species differences exist, as in the cow and sheep IGF-I receptors are expressed in the mature oocyte (Watson et al., 1994; Winger et al., 1997) but in the mouse, are not expressed until the blastocyst stage (Schultz and Heyner, 1993). Therefore, it is unknown whether IGF-I can interact with the porcine embryo and whether IGFBP–4 may be regulating the effects of IGF-I in autocrine or paracrine interactions with the oviduct epithelium. Nevertheless, we have shown that IGF-I in oviduct plasma and in oviduct flushings, and IGFBP-4 mRNA expression in oviduct tissue, are differentially affected by feed restriction and insulin treatment, thus suggesting a possible role of the IGF system in the oviduct mediating nutritional effects on embryonic survival.

In conclusion, this is the first report investigating the effects of previous feed restriction and insulin treatment on the oviduct environment in gilts. Feed restriction in the second week of the estrus cycle resulted in a lower E:P ratio in oviductal plasma, reduced levels of total protein and total protein concentration, IGF-I concentration, POSP abundance and concentration in oviduct flushings, and lower levels of POSP and IGFBP-4 mRNA. These differences are considered to represent a more advanced oviductal environment, driven by the very different steroid milieu created in these animals, and demonstrate the potential for the oviduct to play a role in reduced embryonic survival in this nutritional model. Although insulin treatment did not appear to have counteracted the effects of feeding at the oviductal level, it may have acted through other mechanisms, such as enhanced luteal function to advance the oviductal environment with respect to time after ovulation, as evidenced by changes in POSP abundance and mRNA expression, and IGFBP-4 mRNA expression.

Table 6.1. Least Square Means (± SE of LSM) of peripheral and oviductal plasma progesterone, estradiol and IGF-I concentrations and E:P ratio at surgery. Data from 10 gilts per treatment are represented in this table.

	Treatment[1]		
Parameter	RH	HR	HR + I
Peripheral plasma			
Progesterone (ng/ml)	1.89± 0.25	1.80 ± 0.27	1.64 ± 0.25
Estradiol (pg/ml)	1.16 ± 0.11	0.86 ± 0.12	1.15 ± 0.11
E:P Ratio	0.87 ± 0.08	0.87 ± 0.08	0.84 ± 0.08
IGF-I (ng/ml)	172.9 ± 7.6^{a}	127.9 ± 7.1^{b}	134.4 ± 5.9^{b}
Oviductal plasma			
Progesterone (ng/ml)	52.5 ± 17.0	39.3 ± 18.7	56.1 ± 17.2
Estradiol (pg/ml)	11.1 ± 2.3^{a}	1.51 ± 2.3^{b}	4.8 ± 2.1^{ab}
E:P Ratio	0.34 ± 0.06^{a}	0.19 ± 0.06^{b}	0.14 ± 0.06^{b}
IGF-I (ng/ml)	147.2 ± 6.5^{a}	121.4 ± 7.0^{b}	127.4 ± 6^{b}

[1] RH: gilts feed restricted from d 1 to d 7 of the estrous cycle, then a high plane of feeding from d 8 to the onset of estrus; HR: fed a high plane of feeding from d 1 to d 7 of the estrous cycle, feed restricted from d 8 to d 15, then returned to high plane of feeding until onset of estrus; and HR+I: gilts fed as HR gilts but insulin treated from d 8 to d 15.

^{a,b} Treatment differences (P<0.05) within row are indicated with different letter superscripts.

Table 6.2. Least Square Means (± SEM) of oviduct flushing components across treatments (n=10 gilts per treatment).

Treatment[1]	RH	HR	HR+I
Oviduct flushings			
Volume recovered (ml)	3.60 ± 0.11	3.49 ± 0.10	3.72 ± 0.10
Protein concentration (ng/ml)	240.89 ± 16.9^{a}	167.1 ± 18.5^{b}	162.0 ± 17.0^{b}
Total Protein (ng)	899 ± 74.5^{a}	569.5 ± 69.0^{b}	590.5 ± 64.3^{b}
IGF-I concentration (ng/ml)	11.17 ± 0.56^{a}	9.53 ± 0.64^{ab}	8.39 ± 0.55^{b}
POSP abundance (ratio)	2.69 ± 0.20^{a}	2.41 ± 0.23^{b}	1.59 ± 0.2^{c}
POSP concentration (abundance/ml)	604.5 ± 53.5^{a}	332.3 ± 58.8^{b}	202.6 ± 54.0^{b}
Oviduct tissue			
POSP mRNA abundance	10.58 ± 0.88^{a}	5.55 ± 0.90^{b}	6.39 ± 0.82^{b}
IGFBP-4 mRNA abundance	1.79 ± 0.19^{a}	0.80 ± 0.22^{b}	2.26 ± 0.22^{a}

[1] RH: gilts feed restricted from d 1 to d 7 of the estrous cycle, then a high plane of feeding from d 8 to the onset of estrus; HR: fed a high plane of feeding from d 1 to d 7 of the estrous cycle, feed restricted from d 8 to d 15, then returned to high plane of feeding until onset of estrus; and HR+I: gilts fed as HR gilts but insulin treated from d 8 to d 15.

^{a,b} means within row with different letters differ (P<0.05)

Figure 6.1. Relationship between IGF-I concentrations in oviductal plasma and flushings.

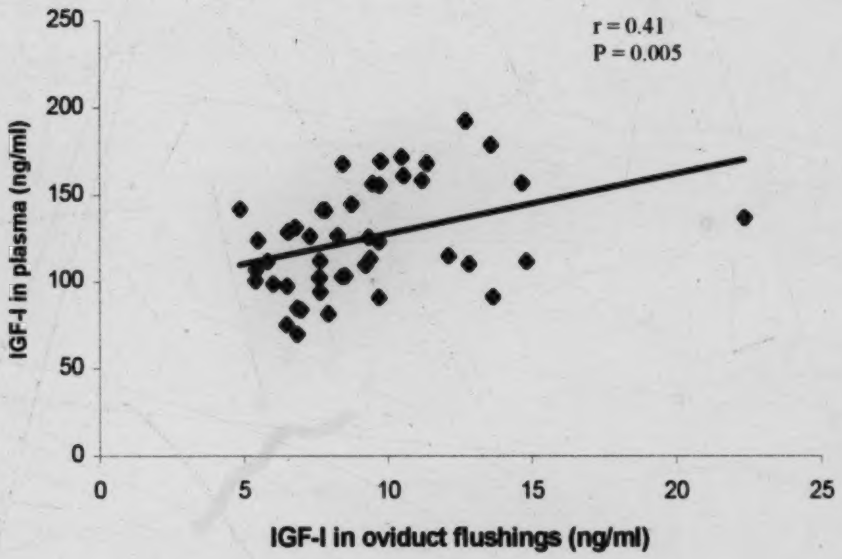


Figure 6.2. **A)** Representative Western blot of POSP protein abundance. Positive control (+) is shown and treatments are labeled (HR, RH, HR+I). R and L oviduct flushings within animal are indicated on the figure also. **B)** POSP mRNA abundance on Northern blots. 28S band on gel is shown in panel below for reference.

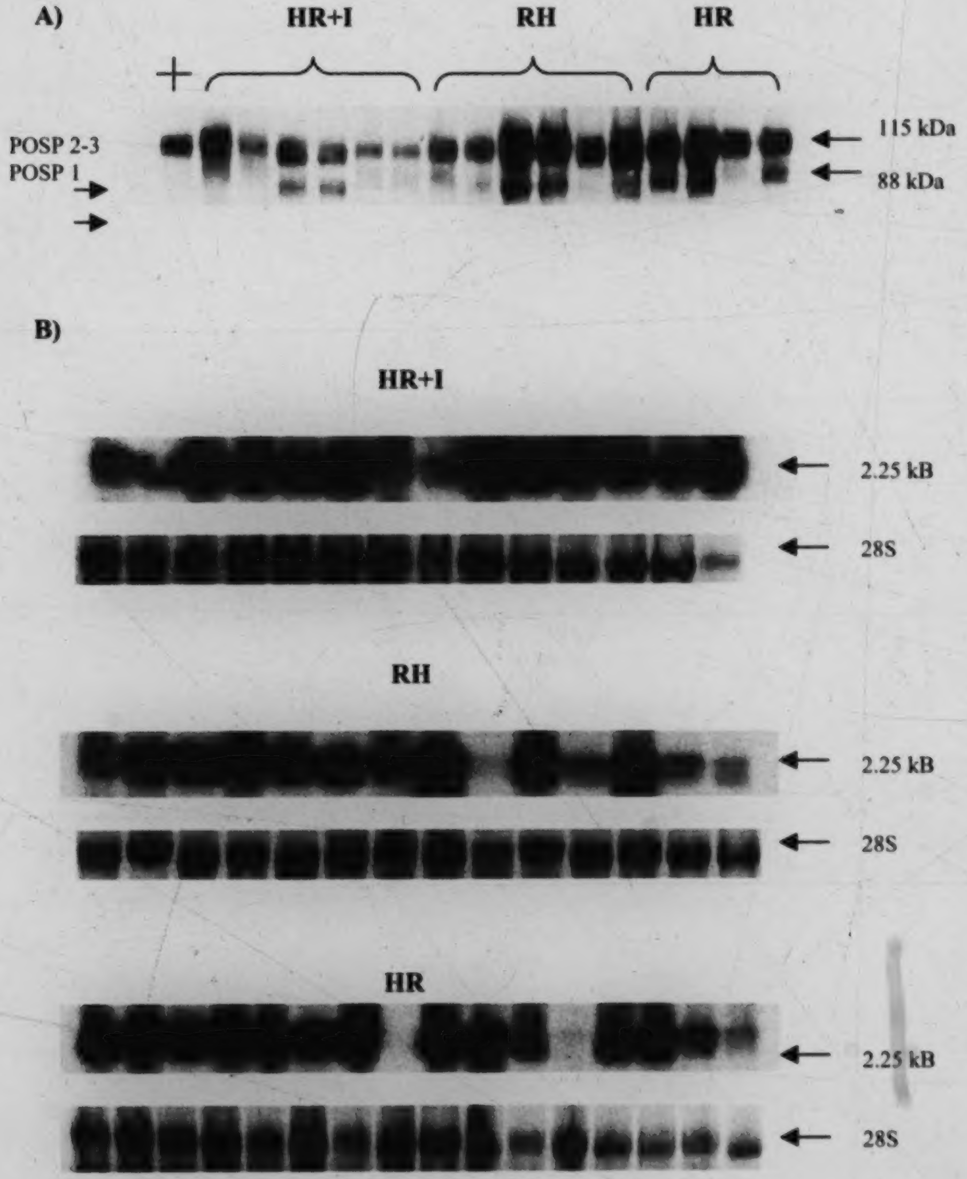

A)
HR+I
RH
HR
+
POSP 2-3
POSP 1
115 kDa
88 kDa
B)
HR+I
2.25 kB
28S
RH
2.25 kB
28S
HR
2.25 kB
28S

Figure 6.3. Relationship of POSP mRNA abundance to time after ovulation across treatments (7 gilts for RH, 7 gilts for HR, and 8 gilts for HR+I).

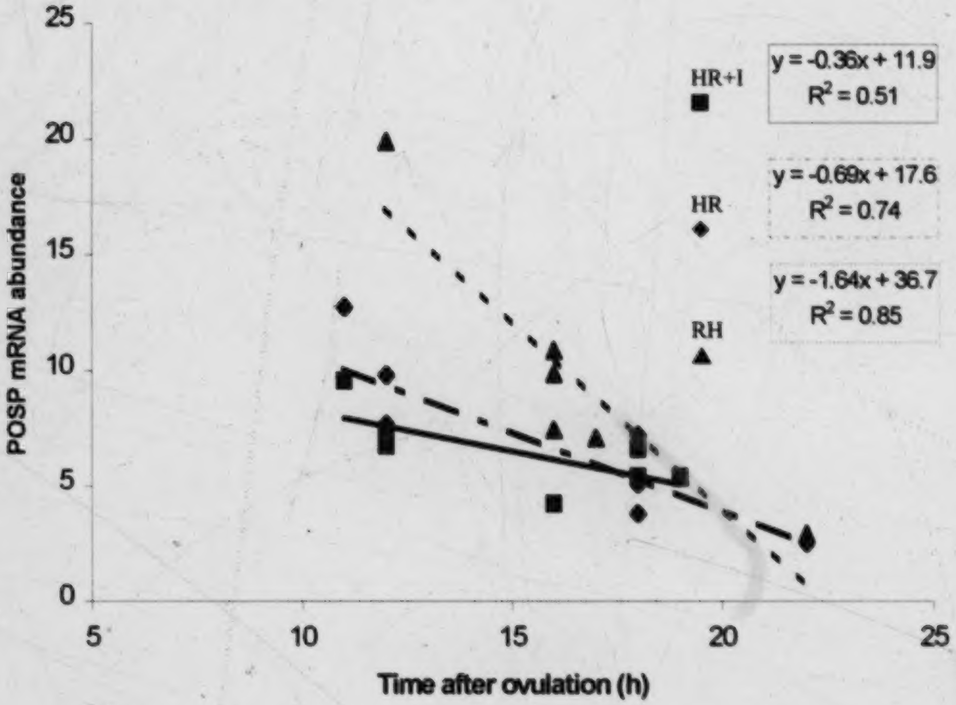
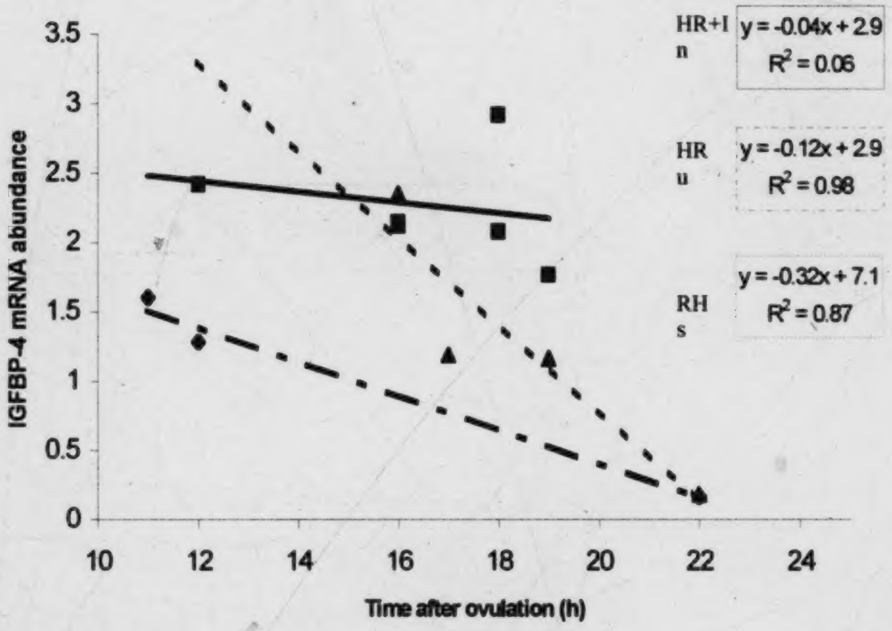


Figure 6.4. Relationship of IGFBP-4 mRNA abundance to time after ovulation in RH (n=4 gilts), HR (n=3 gilts), and HR+I (n=5 gilts).



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GENERAL DISCUSSION AND CONCLUSIONS

The mammalian oviduct has been long thought of as an important conduit for the passage of gametes and embryos, providing the appropriate nutrients, growth factors, and environment for these critical early stages (see Mastroianni and Komins, 1975; Bavister, 1988; Leese, 1988; Ellington, 1991; Menezo and Guerin, 1997). However, recent evidence shows that through administration of exogenous steroids (Day and Polge, 1968; Binelli et al., 1999; Kamishita et al., 1999), and surgical manipulation (Sultan and Bedford, 1996), alterations in sperm transport, fertilization, early embryonic development in vitro, and in the pattern of oviduct protein secretion occurs. In our laboratory, nutritional models have been established whereby alterations in the pattern of feeding of gilts, and sows during lactation, resulted in reduced embryonic survival (Jindal et al., 1996; Zak et al., 1997a; Almeida et al., 2000a). This effect on embryonic survival is in part mediated by alterations in progesterone concentrations in the first few days of pregnancy (Pharazyn et al., 1991; Pharazyn, 1992; Jindal et al., 1997), when the cleavage stage embryos are still in the oviduct. Therefore, the overall objective of these studies was to determine whether the oviduct could be involved in nutritionally induced embryonic survival.

A key aspect of the oviduct in the pig is that it is exposed to high concentrations of ovarian steroids through the function of the sub-ovarian countercurrent system. In Chapter 2, we demonstrated that the concentrations of progesterone immediately after ovulation were higher in the oviductal circulation than in the peripheral circulation. We also found that at a time when peripheral concentrations of progesterone were rising with respect to time after ovulation, progesterone concentrations were already high in the oviductal circulation. This suggested that if the oviduct is involved in nutritionally induced embryonic survival, then changes in peri-estrous estrogen and progesterone profiles would exert their effects on the oviduct during the periovulatory period. In contrast, the oviductal environment would probably not mediate nutritionally induced effects on embryonic survival, when treatments were imposed after ovulation. As a result, we utilized a model where nutritional treatments are imposed during the estrous cycle, and hence could potentially alter follicular development, in order to elucidate whether the oviductal environment was also affected and could potentially mediate embryonic survival.

Furthermore, our findings in Chapter 2 suggested that in order to determine if the oviductal environment was affected by previous nutritional treatment, one should examine changes in the oviductal environment before 12 to 24 hours after ovulation, which was the sampling time used in these studies. This was a concern for the following studies (Chapters 5 and 6), because they were part of larger collaborative experiments where the opportunity to obtain oviduct fluid samples was fixed at 12-20 h after ovulation, and this might not have been ideal for observing changes in the oviductal environment between the treatment groups. A shorter interval between ovulation and time of the initial rise in progesterone, and higher progesterone concentrations at 48 to 96 h after the onset of estrus, associated with prior nutritional manipulation during follicular development (Zak et al., 1997b; Almeida, 2000; Almeida et al., 2000a), suggests that follicular maturation and associated steroidogenesis, affects subsequent luteinization. These interactions had also been postulated by Hunter and Weisak (1990) and Foxcroft (1997). Evidence to support this hypothesis has been presented by Zak et al., (1997b) and Mao et al., (2000), showing that with nutritional treatments that reduce embryonic survival, follicular and oocyte maturation, and luteal function, were compromised. In other words, progesterone concentrations could be used as an indicator of the previous follicular quality. Therefore, we rationalized that if estrogen concentrations before ovulation affected the oviductal environment, these effects would still be evident in the immediate post-ovulatory period.

Therefore, to properly interpret the results on oviductal function obtained from a discrete window of time after ovulation, it was critical to first characterize components of the oviductal fluid over the peri-estrous period. Although many studies have examined oviductal protein concentrations, protein secretion, and oviduct fluid formation in relation to the estrous cycle, none compared discrete changes during the peri-estrous period. Furthermore, no studies to date have examined the oviductal environment with respect to local circulating estrogen and progesterone concentrations in vivo, nor in relation to ovulation. This is critical in our opinion, to establishing specific relationships between hormonal profiles and the oviduct environment, especially when one considers that the oviduct is responsive to ovarian steroid concentrations, and ovulatory products (Hunter, 1984; Mburu et al., 1996; Hunter, 1995; Sultan and Bedford, 1996). Also, we have confirmed that there is a large variation between the onset of estrus and time of ovulation in gilts (Almeida et al., 2000b), strongly suggesting that in order to observe differences in the oviductal environment, data should be considered in relation to ovulation, not to onset of estrus. Furthermore, the estrus period is characterized by a dynamic interaction between declining estrogen concentrations and increasing progesterone concentrations, suggesting that discrete changes in the oviductal environment are necessary to accommodate the diverse events occurring within the oviduct at this time. In addition, we know that the oviduct is exposed to high concentrations of progesterone in advance of a peripheral rise, as evidenced by Eiler and Nalbandov (1977), Hunter et al. (1983) and Chapter 2. In Chapter 3, we have effectively shown that protein concentrations, POSP mRNA and protein abundance, and IGF-I concentrations changed in relation to time of ovulation. Furthermore, these changes were associated with either progesterone concentrations or the E:P ratio. This study is the first to characterize such changes, and demonstrate the importance of the time of ovulation on the oviductal environment in the pig.

Interestingly, steady-state POSP mRNA expression persisted 24 hours after ovulation, and POSP protein abundance remained high until after that time, suggesting that POSP is being actively synthesized and secreted despite low estrogen, and high progesterone, concentrations. As discussed earlier, studies have shown that the synthesis and secretion of POSP occurs in response to exogenous estrogen treatment (Buhi et al., 1992), and were present throughout the estrous period, during which estrogen concentrations are supposedly high (Buhi et al., 1989).

This prompts the speculation that the magnitude of the estradiol peak may prolong the presence and abundance of POSP in oviduct fluid after ovulation, benefiting the early cleaving embryo. In support of this, in Chapter 6, we showed that POSP mRNA and protein abundance were higher in the RH gilts that Almeida (2000) reported as having higher peak estradiol concentrations before ovulation. Also, POSP protein abundance in the oviduct ipsilateral to the remaining ovary (INT) was higher than in the contralateral (OVX) oviductal flushings in unilaterally ovariectomized gilts, suggesting that in the oviduct exposed to higher estradiol concentrations, POSP protein abundance was higher before ovulation and/or persisted longer after ovulation.

One of the most exciting results of this series of studies was the conclusion that the sub-ovarian countercurrent system has functional significance in vivo, and that the magnitude of ovarian steroid concentrations changes the oviductal environment as shown in Chapter 4. Indeed the presence of peripheral concentrations of steroids in the OVX oviductal circulation, compared to the 10-fold higher concentrations in the INT oviductal circulation, and resultant differences in oviductal protein concentrations and POSP abundance, strongly suggest that the oviduct is locally regulated by ovarian steroids. Furthermore, these results demonstrate that the in vivo oviductal environment cannot be accurately regulated by exogenous steroid administration to ovariectomized pigs. Although peripheral concentrations were enough to synthesize and secrete some POSP, the lack of high concentrations of ovarian steroids in the OVX oviductal circulation affected the POSP abundance in oviduct flushings.

Also, although studies in cows have shown that the oviductal environment (Binelli et al., 1999) and its ability to sustain in vitro embryo development (Kamishita et al., 1999), is locally regulated by ovarian steroids, this is in a typically monovulatory species. We have been able to show that in intact gilts, each oviduct is independently regulated by local oviductal concentrations of steroids. In Figure 5.3, we presented evidence that POSP abundance was negatively associated with oviductal progesterone concentrations in that oviduct, independent of animal.

Furthermore, the oviductal circulation has a different E:P ratio than peripheral plasma (Chapters 4, 5 and 6), and thus creates a unique steroid milieu. Although we have shown differences in concentrations of total protein, IGF-I and POSP in oviduct flushings in relation to estrogen and progesterone concentration, there are many other aspects of oviduct function that will be simultaneously affected. The regulation of oviduct function is very unique and complex, and the evidence in this thesis has only examined a few of the components in oviduct flushings. Ovarian steroids regulate ovarian and oviductal blood flow (Magness et al., 1983), fluid synthesis (Bishop, 1956; Sutton et al., 1984; Wiseman et al., 1992), contractility (Borda et al., 1980), as well as oviduct protein synthesis and secretion (Lippes et al., 1991; Sutton et al., 1984; Buhi et al., 1989; Gandolfi et al., 1989). Although the peripheral circulation is considered a reflection of ovarian steroidogenesis, there are differences between the peripheral and oviductal circulation in the immediate post-ovulatory period. Therefore, when examining the oviductal environment in future studies, the importance of determining the concentrations of ovarian steroids in the oviductal circulation should not be overlooked.

Overall, some conclusions can be made in regards to the regulation of the oviductal environment by ovarian steroids. A complex interaction exists between absolute concentrations of ovarian steroids and the E:P ratio in regulating the oviductal environment, and although they are linked, we have evidence to suggest they influence different aspects of oviduct function. A good example is in the unilateral ovariectomy model, where a higher protein concentration is present in the OVX oviduct, representative of active fluid synthesis, and in the same oviduct POSP abundance is lower. In contrast, in intact animals, a higher protein concentration in oviduct flushings is associated with a higher POSP abundance (Chapter 3, Chapter 5, Chapter 6). Taken together, these data suggest that oviductal fluid dynamics, and therefore, protein concentrations, IGF-I and POSP concentrations, are influenced by the E:P ratio, whereas POSP abundance is influenced by absolute concentrations of ovarian steroids. In support of this, the measurement of POSP abundance is independent of oviduct fluid dynamics, and tends to be related to progesterone concentrations (Chapter 3, Chapter 4, Chapter 5). In contrast, all concentration measurements in oviduct flushings tend to be related to the E:P ratio (Chapter 3, Chapter 4, Chapter 5).

Lastly, although few authors have postulated that the oviductal environment is involved in nutritionally induced embryonic survival (Ahmad et al., 1995; Jindal et al., 1997; Mburu et al., 1998), we have been the first to provide direct evidence of changes in the oviductal environment in association with previous nutritional treatment. In Chapters 5 and 6, when gilts were restricted in the second week of the estrous cycle, during the period of follicular recruitment and growth, the subsequent protein, IGF-I, and POSP concentrations were lower, and POSP mRNA and protein abundance were reduced. This treatment group also exhibited a lower pre-ovulatory estradiol peak and a slower rise in peripheral progesterone concentrations (Almeida, 2000). In addition, Mao et al. (2000) reported that the luteal cells from these gilts did not respond to LH in vitro, providing evidence that nutritional effects on follicular development extend into the post-ovulatory period. Our results from Chapter 6, build on these data and strongly support the hypothesis that the same mechanisms that alter follicular steroidogenesis, will subsequently affect the oviductal environment. An interesting result from this study is the differential effect of exogenous insulin on the oviductal environment. The insulin treatment appeared to advance the environment in relation to ovulation, and reduce POSP abundance compared to feed-restricted groups. Mao et al. (2000) also reported similar findings in their studies with luteal cells from these insulin-treated gilts. They concluded that the steroidogenic capacity of follicles, and subsequent newly formed corpora lutea, as determined by measuring mRNA expression in key steroidogenic enzymes and progesterone production in vitro, was advanced in insulin treated gilts. Similarly, there were alterations in mRNA expression profiles in POSP and IGFBP-4 in the insulin treated gilts in Chapter 6. How this advanced oviductal environment may be beneficial to the developing embryo still remains to be determined.

One of the most important questions that all of this evidence raises, is whether the observed changes in the oviductal environment in Chapter 6 are enough to adversely affect embryonic survival. Although we have shown that circulating ovarian steroids influence the oviduct environment, are subtle differences in protein concentrations and POSP concentrations enough to compromise embryonic development? Even if there are reduced concentrations of POSP and IGF-I in oviductal fluid, the amounts present may be adequate for optimal fertilization and early embryonic development to occur. If we assume that other aspects of oviduct function and components of oviduct fluid are also adversely affected, are the magnitude of these changes of physiological significance? This perhaps is more critical for embryonic survival, because ovarian steroids may adversely influence contractility, affecting gamete and embryo transport (Hunter et al., 1983), and essential environmental factors such as pH (Nichol et al., 1997). It would be much simpler if we showed that a factor was present in one treatment and absent in another; however, this is not the case.

The answer may lie in the timing of these changes in the oviduct environment in relation to events occurring within the oviduct during the peri-ovulatory period. Soede et al. (1994) showed that a shorter interval between peak estradiol and the time of the rise in progesterone concentrations was associated with higher embryonic survival in sows, suggesting that the timing of the rise and fall of the respective hormones is critical for embryonic survival. This is also supported by other nutritional studies reporting that higher progesterone concentrations, and the timing of progesterone concentrations are associated with higher embryonic survival (Ashworth, 1991; Pharazyn et al., 1991; Pharazyn, 2000; Jindal et al., 1996; Almeida et al., 2000a; van den Brand et al., 2000). Therefore, alterations in steroid hormone profiles in the oviductal circulation may adversely affect priming of the oviduct, and the timing of changes in the oviductal environment in relation to ovulation.

One important consideration is that the same factors that affect the oviductal environment may also be affecting oocyte quality. Because a heterogeneous population of pre-ovulatory follicles exists in the gilt (Foxcroft and Hunter, 1985; Grant et al., 1989), and oogenesis directs embryogenesis (Xie et al., 1990), some degree of embryonic diversity is unavoidable. Nutritional restriction during follicular development may have created a less mature population of oocytes, as shown by Zak et al., (1997b) and Yang et al. (2000), thus compromising embryonic quality. The oviduct then may contribute to the asynchrony between embryos and the uterus (see reviews by Pope, 1988; Pope, 1990) by augmenting embryonic diversity during their residence in the oviduct, resulting in increased peri-implantation loss. This is likely the impact of the oviductal environment on embryonic survival, as any one factor is probably not missing from the oviduct fluid entirely in these treatments. In addition, if embryonic quality is indeed compromised as a result of nutritional treatment, subtle alterations in the oviductal environment may contribute to reduced embryonic viability through asynchronous development of the oviduct and embryo.

The results from this series of studies presents us with the opportunity to consider future studies designed to further elucidate whether nutritionally-induced embryonic survival is mediated by changes in the oviductal environment. It is necessary to set up such experiments to hold all other factors constant so that differences in oviduct function to be the only manipulated variable. Firstly, the use of littermates, as in Chapter 5 and 6 is essential, as we observed littermate effects in every parameter measured, especially in steroid hormone concentrations. Secondly, as resultant embryonic quality from nutritional treatment may confound the results, embryonic quality must be somehow held constant. One way to achieve this in vitro would be to culture generic embryos in co-culture with oviduct fluid, or oviductal cells obtained from the different nutritional treatments. Although it would not be possible to create the critical changes of the oviductal environment with respect to time in this situation, the benefit of this design would be that the oviduct environment would be the only factor directly affecting embryonic development in vitro. Another option would be to do an embryo transfer immediately after ovulation, by inserting generic oocytes into the oviducts of nutritionally manipulated gilts, therefore allowing for treatment-dependent changes to occur in the oviduct environment. The inherent problem with this method is that if the embryos are not removed before they enter the uterus, the uterine environment may also mediate changes in embryonic survival. Finally, as these studies only examined the oviductal environment after ovulation, it would be ideal to determine if similar changes were evident in the pre-ovulatory period, as our data suggest that they would. The design of an experiment to effectively answer this complex problem would undoubtedly require a combination of in vitro techniques and surgical embryo transfer at different time points throughout the peri-ovulatory period.

Also, specific glycoprotein secretions of the oviduct, such as POSP, are thought to play an important role for the gametes and developing embryos and have been the subject of many reviews (Hunter, 1994; Gandolfi, 1995; Murray et al., 1995; Nancarrow and Hill, 1995; Abe, 1996; Buhi et al., 1997; Verhage et al., 1998). Recent evidence demonstrates that the addition of these glycoproteins to fertilization medium improves fertilization in the cow (Martus et al., 1998) and sperm-egg binding in the hamster (Schmidt et al., 1997). Also, growth factors, such as IGF-I, are thought to be important for development of the pre-implantation embryo (Schultz and Heyner, 1993; Chegini, 1994; Einspanier et al., 1997; Heyner, 1997; Kaye, 1997). Our evidence supports potential roles of POSP and IGF-I in embryonic development as POSP expression and POSP and IGF-I secretion are influenced by steroids and nutritional manipulation, and may be a potential factor in embryonic survival in the pig. Further studies could continue to address this question, and a variety of methods can be implemented to achieve this. In vitro matured and fertilized embryos can be incubated in varying levels of POSP or IGF-I, and then embryonic survival can be monitored upon transfer into the uterus. The most elaborate of all experiments would be to create transgenic pigs with the POSP gene knocked out, and the impacts of its disappearance on embryonic survival could be readily distinguished.

Lastly, one other important component of this system not to be overlooked in the role of the oviduct on embryonic survival, is that alterations in the timing of changes in ovarian steroid hormones and in oviduct secretions may adversely affect sperm maturation and transport, thus leading to changes in fertilization. Almeida (2000) demonstrated that the fertilization rate in gilts feed-restricted during the second week of the cycle was slightly reduced, so fertilization rate may be a factor in the overall measurement of nutritionally-mediated embryonic survival. As discussed earlier, changes in the timing of the rise of progesterone may adversely affect release of sperm from the sperm reservoir and sperm transport to the fertilization site (reviewed by Hunter , 1995). If we assume that alterations in estrogen and progesterone are not pronounced enough to cause inadequate numbers of spermatozoa at the fertilization site, differences in timing or in amounts of oviductal secretions may affect sperm maturation and capacitation. In vitro experiments have shown that co-culture of sperm with oviductal cells reduces polyspermy rates (Dubuc and Sirard, 1995), maintains sperm viability and fertilizing ability (Pollard et al., 1991; Nichol et al., 1997; reviewed by Smith, 1998, Yao et al., 1999), and increases fertilization rates (Way et al., 1997). Furthermore, researchers have shown that specific factors secreted by the oviduct may be responsible for the beneficial effects of the oviductal epithelium on sperm viability (Abe et al., 1995; Boatman and Magnoni, 1995; Verhage et al., 1998; Yao et al., 2000), and that the ability of the oviduct to maintain viability of sperm is enhanced by 17ß-estradiol (Boquest and Summers, 1999). This is an area that can also be looked at in future studies, as sperm can be recovered from oviducts immediately before fertilization in pigs known to have experimentally induced differences in embryonic survival, and can be examined in vitro for differences in sperm viability and fertilizing ability using sperm-egg binding techniques.

In conclusion, the results presented in this thesis provide convincing evidence that the oviduct is influenced by local ovarian steroids, and as such, is potentially involved in embryonic survival. Although we have only examined the regulation of a few potentially embryotrophic factors in the oviduct, these studies implicate the alteration of other factors and oviduct function in general, which may also contribute to embryonic loss in the pig. Future studies arising from the results in this thesis may be able to answer whether the oviduct can mediate nutritionally-induced embryonic survival, and confirm the roles of oviduct secretions in embryonic development.

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APPENDIX 1 Dietary compositions as formulated of weaner, grower, and gestation rations used.

Ingredients %	Weaner	Grower	Gestation
Wheat	26.8	-	-
Barley	9.8	75.9	86.0
Soybean meal	15.8	12.0	4.0
Canola meal	.	6.1	4.5
Oat Groat	19.5	-	-
Fish meal	4.2	-	-
Lysine	0.2	0.2	-
Whey	15.6	-	-
Limestone	1.0	1.1	1.6
Dicalcium phosphate	1.2	0.9	1.4
Salt	0.5	0.4	0.5
Oil	4.3	2.3	1.0
Premix[a]	1.1	1.1	1.0
Chemical analysis			
Digestible energy, kcal/kg	3,544	3,314	3,120
Crude Protein %	20.15	17.3	13.75
Calcium %	0.86	0.72	0.93
Total Phosphorus %	0.75	0.66	0.70
Lysine %	1.30	0.99	0.56
Methionine + Cysteine %	0.66	0.55	0.44
Threonine	0.76	0.61	0.47

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[a] Premix composition per kilogram of diet: Weaner and gestation diets- Vitamin A 10,000 IU, vitamin D 1,000 IU, vitamin E 80 IU, vitamin K 2 mg, vitamin B12 30 µg, riboflavin 12 mg, niacin 40 mg, pantothenic acid 25 mg, choline 1,000 mg, biotin 250 µg, ethoxyquin 5 mg, iron 150 mg, manganese 12 mg, zinc 120 mg, copper 20 mg, iodine 200 µg, selenium 300 µg. Grower Diet- Vitamin A 5,000 IU, vitamin D 500 IU, vitamin E 40 IU, vitamin K 2 mg, vitamin B12 30 µg, riboflavin 12 mg, niacin 40 mg, pantothenic acid 25 mg, choline 300 mg, biotin 150 µg, ethoxyquin 5 mg, iron 150 mg, manganese 12 mg, zinc 100 mg, copper 20 mg, iodine 200 µg, selenium 300 µg.